THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XII JUNE, 1931 No. 3

AGGLUTININS OF TICK-BITE FEVER AND SPORADIC TYPHUS IN SOUTHERN AFRICA IN MAN AND IN GUINEA-PIGS.

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Forwarded for publication March 18th, 1931.

WE claim that in Southern Africa there exists, apart from typhus, a separate *typhus-like* disease, which should be called tick-bite fever, after Nuttall (1911).

Tick-bite fever and typhus are often confused. Most publications are not clear on this point (McNaught, 1911 ; Anderson, 1925 ; Jewell and Cormack, 1930).

We shall report elsewhere about clinical differentiation of the two diseases ; here we shall deal with serological differentiation.

Formerly we have shown that tick-bite fever can be transmitted to guinea-pigs, that such guinea-pigs can react with typical fever-curves, and that in their brains certain nodules can be found (1930, *a*, *b*).

TECHNIQUE OF AGGLUTINATION.

All tests were done in a serum dilution of 1 : 100. Serums were used unheated and without preservatives. Live bacillary suspensions were used of the following strains : *B. proteus* OX 19, OX 2, and X Kingsbury, and *B. agglutinabilis* Wilson (see Wilson, 1927, 1929). We strictly followed the instructions of Felix (1924) as regards growing cultures, reading results, etc. Our observations refer to O-agglutination only. For a detailed discussion of the antigenic construction of *B. proteus* we refer to Felix's recent publication (1931).

Our results, as exemplified in Table I, warrant the following conclusions :

1. Our *weak* agglutination results resemble those in tsutsugamushi disease recorded by Fletcher (quoted by Felix, 1931) and by Felix (1931), and those recorded in Rocky Mountains spotted fever by Spencer and Maxcy (1930), and also those found in the " fièvre exanthématique " of Marseilles by various French authors. We ascribe significance to our weak positive findings because of our numerous completely negative findings in various other diseases.

AGGLUTINATION REACTIONS OF TICK-BITE FEVER PATIENTS.

Typical examples are given in Table I.

TABLE I.—*Showing Typical Agglutination Reactions of Tick-bite Fever Patients.*

Patient.	Day of disease.	Days serum kept.	OX 19.	OX 2.	X Kingsbury.	*B. agglutinabilis.*
1.	4th	1	−	−	+	(+)
	11th	0	(+)	+(+)	+	−
	11th	7	+(+)	++	+	+
	20th	0	+	(+)	+	(+)
	20th	13	(+)	(+)	+(+)	−
	35th	4	+	+(+)	+(+)	(+)
	35th	19	++	+(+)	+(+)	−
	4 months later	8	+	(+)	+(+)	(+)
2.	3rd	3	−	−	+	(+)
	9th	3	+(+)	(+)	(+)	+
	9th	18	+(+)	++(+)	+	−

2. Tick-bite fever produces agglutinins for all three X-strains, thus again resembling Rocky Mountains spotted fever (Spencer and Maxcy, 1930).

3. Early in the disease there are no agglutinins. The best time to find agglutinins is in convalescence. This has been noticed by Fletcher and co-workers in tropical typhus (1929), by Olmer and Olmer in the Marseilles fever (1928), and by Plazy in the fever of Toulon (1930), but we wish to specially emphasize it.

4. Serums of tick-bite fever gain in agglutinative power simply by standing. This phenomenon of what might suitably be called " temporary inhibition " was specially noticeable in typhus-serums from Asiatic Turkey, in contrast to serums from Europe (Felix, 1917). One can also see it in typhoid serums (Pijper, 1923). Kraus (1929) simply called it an unexplainable phenomenon, but of great practical importance. We have been so greatly impressed by the reality of the phenomenon that we avoid doing agglutination reactions with fresh serums, especially in tick-bite fever, and we suggest that the contradictory results recorded by various authors in typhus-like diseases are due to this " temporary inhibition." For an example see Burnet and Durand on " fièvre boutonneuse " (1929). Heating the serum might help (Felix, 1930), but would also damage O-agglutinins. In numerous normal serums we have never found traces of O-agglutinins develop, however long we kept them.

AGGLUTININS OF GUINEA-PIGS INFECTED WITH TICK-BITE FEVER.

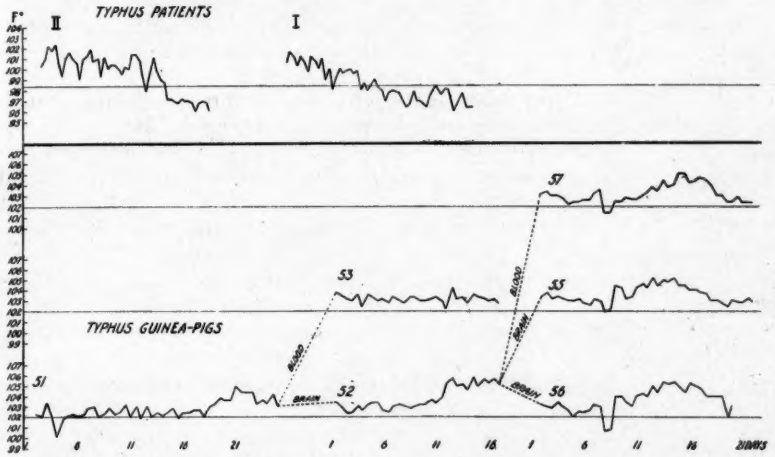
We have performed agglutination reactions with the blood-serums of all the guinea-pigs we have infected with tick-bite fever, irrespective of the absence or presence of a temperature curve. Altogether we have brought ten different strains from patients into guinea-pigs. In most instances the second or third passage, in some instances the fourth or fifth animal passage was reached. We possess agglutination records of 45 infected guinea-pigs. All of them gave positive results with X Kingsbury, in a dilution of 1 : 100, in a strength that varied from + to +++. Tests with OX 19, OX 2 and *B. agglutinabilis* were invariably negative. Controls with normal guinea-pigs' serum were always negative. Here also the phenomenon of " temporary inhibition " was very

pronounced; the best time to find agglutinins was also during convalescence, and some animals kept their agglutinins for several months after infection.

AGGLUTINATION REACTIONS OF TYPHUS PATIENTS.

We had an opportunity of examining three cases of what we on clinical grounds called typhus; they occurred sporadically. One of the patients died; the post-mortem corroborated our view. Other feverish diseases could be excluded by the usual methods. The temperature curves of two cases are given in the figure.

All three cases showed agglutinins for OX 19 and OX 2, and also some for *B. agglutinabilis*, but agglutinins for X Kingsbury were totally absent. In



two of the three cases the titre for OX 19 and OX 2 was determined: it reached 1:400 in the one and 1:800 in the other case. In this respect, then, our cases of sporadic typhus resembled European typhus, and differed from American typhus (see Felix, 1931).

AGGLUTININS OF GUINEA-PIGS INFECTED WITH SPORADIC TYPHUS.

We infected guinea-pigs with the blood of the fatal case of our typhus patients. The strain proved easily transmissible from guinea-pig to guinea-pig and most animals reacted with the classical fever curve. The figure illustrates some of these experiments.

The results of agglutination reactions with blood-serum from such guinea-pigs are given in Table II.

It is generally recognized that guinea-pigs infected with typhus do not develop agglutinins for X 19, or very exceptionally, and in very small quantities. We find (Table I) that such animals, at least in South Africa, develop agglutinins for X Kingsbury. As far as we know, others have not tried X Kingsbury on infected guinea-pigs.

TABLE II.—*Showing the Agglutination Reactions of Guinea-pigs Infected with S. African typhus ; the Numbers of the Guinea-pigs refer to the Figure.*

Guinea pig's number.	Day after infection.	Days serum kept.	OX 19.	OX 2.	X Kingsbury.
51	21	1	−	−	+++
52	17	5	−	−	(+)
53	17	2	−	−	+(+)
54	25	2	−	−	+(+)
55	21	1	−	−	(+)
55	21	6	(+)	(+)	++(+)
56	19	4	−	−	+(+)
56	19	9	−	−	++
57	21	1	−	−	−
57	21	6	−	−	+(+)

Felix (1930, 1931) has tabulated what is known so far about agglutination reactions in typhus and typhus-like diseases. At that time nothing was known in this respect about typhus-like fevers in Southern Africa. Of the typhus-like disease in Kenya, the publications of Anderson (1925) and of Jewell and Cormack (1930) only give information about agglutination reactions with the strain X 19. Our publication is intended to fill the gap, and bring tick-bite fever in line with typhus-like diseases in other lands.

SUMMARY.

The typhus-like disease in Southern Africa, which we propose to call tick-bite fever, produces agglutinins for *B. proteus*, OX 19, OX 2 and X Kingsbury.

Typhus in South Africa produces agglutinins for OX 19 and OX 2, but not for X Kingsbury.

Guinea-pigs infected with either tick-bite fever or typhus develop agglutinins for X Kingsbury, but not for OX 19 nor for OX 2.

All these agglutinins appear to become more active if one lets the serums stand for some days. This phenomenon of " temporary inhibition " may explain certain discrepancies observed in agglutination reactions with X-strains.

In tick-bite fever agglutinins appear late in the disease.

We thank Dr. Felix for giving us cultures of OX 19, OX 2 and X Kingsbury, and Prof. Wilson for giving us a culture of *B. agglutinabilis*. We also thank Dr. Felix for giving us a copy of the manuscript of his paper, ' Serological Varieties of Typhus Fever ' (1931). Our thanks are also due to Dr. J. McDonald Troup, Dr. W. H. L. Wronsley, and Dr. J. C. Truter, all of Pretoria, for giving us clinical material, and to Dr. R. R. Scott, of Dar-es-Salaam, and Dr. W. G. S. Hopkirk, of Nairobi, for helping us with literature from Kenya.

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THE CAUSE OF INACTIVATION OF THE ROUS SARCOMA FILTRATE DURING INCUBATION.

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Received for publication March 20th, 1931.

GYE and Purdy (1930) showed that although the filtrate from a Rous sarcoma became inactive if it were incubated at 37° C. for 24 hours, the addition of small amounts of hydrogen cyanide caused the filtrate to retain its activity at 37° C. for up to three days. Mueller (1928) had also shown that small amounts of cysteine added to the filtrate preserved its tumour-producing power for about three days under similar conditions. After the addition of cysteine the filtrate was placed either under a layer of vaseline, or in an anaërobic jar. It was possible that this preservation was due either to inhibition of an oxidation, or to some change in the activity of proteolytic enzymes in the filtrate. Grassmann and Dyckerhoff (1930) showed that cysteine and cyanide inhibited the action of ereptic aminopolyase, while they accelerated the action of proteases such as cathepsin. In any studies of the concentration, cultivation or metabolism of a virus, it is useful to know the conditions under which maximum stability may be attained, and also what circumstances and factors are likely to injure it. Consequently it seemed of interest to decide whether the fragility of the Rous virus were due to its inability to resist oxidation, or whether a proteolysis were the inactivating cause. Gye and Purdy (1930) and Mueller (1928) had found that incubation of the filtrate under anaërobic conditions under vaseline, or more strictly, in an anaërobic jar, had very little effect upon

the duration of activity. Hence, if the inactivation is due to oxidation, atmospheric oxygen could play no part in it. Both the reducer and the oxidizer must be contained in the filtrate itself. These experiments have been repeated with the same results.

A series of experiments has been carried out comparing the action of cysteine, sodium hydrosulphite and methylene-blue in air and under anaërobic conditions on the tumour-producing power of the Rous filtrate. Under anaërobic conditions the two former substances maintain a high reduction potential, and an oxidation of another substance could only occur if its reduction potential were higher than that of the added sulphur compound. In the presence of air, however, where the sulphur compound is autoxidizable, a coupled oxidation of other substances may also take place. Thurlow (1925) found that coupled oxidation of lactic acid, nitrites, or β-oxybutyric acid could occur during the autoxidation of cysteine in the presence of a peroxidase, and if inactivation of the tumour-producing system is due to oxidation, it should take place more rapidly in air in the presence of cysteine or sulphite than with the filtrate alone. The following table shows that this is indeed the case. Both cysteine and sodium hydrosulphite preserve the activity of the filtrate under anaërobic conditions, but in the presence of air act as rapid destructors of its activity.

TABLE I.—*Effect of Sodium Hydrosulphite and Cysteine.*

Date.	No. of bird.	Time of incubation in hours.	Control aërobic.	Sulphite aërobic.	Control anaërobic.	Sulphite anaërobic.
22.4.30	474	44	..	..	++	+++
22.4.30	475	44	..	..	(+)	+++
3.5.30	494	22	—	—	—	—
	493	22	—	—	—	++++
22.5.30	539	26	—	—	—	————
	540	26	++	—	++	+++
4.7.30	586	42	..	..	—	++
	589	42	..	..	—	+
27.3.30	429	1	++	—	..	..
	430	1	++	—	..	..
	431	1	++	—	..	..
	432	1	—	++	..	..
	433	1	++++	—	..	..
	434	1	++	—	..	..
	437	24	+++	—	..	..
			Cysteine			
16.7.30	311	24	+++	—	..	..
	313	24	+++	—	..	..

++++ = large tumour. (+) = very small tumour; — = no tumour. Concentration of cysteine or sodium hydrosulphite = 1/1000. pH = 7·4.

In the following experiments a 4 per cent. extract of tumour tissue in saline has been used. This has been prepared by grinding fresh tumour tissue with sand and adding saline until a 4 per cent. suspension was obtained. This was centrifuged thoroughly and usually put through an English Berkefeld

candle. In certain cases where absolute sterility was not essential the spun extract was filtered through a paper-pulp and sand filter, which is known to remove all tumour-cells. At the end of the experiment 1 c.c. or 0·5 c.c. of the differently treated filtrates were injected into the breasts and legs of young Plymouth Rock birds. Comparisons were only made between tumours in the breasts, or between tumours in the legs of the same bird It is impossible to compare the size of tumour in one bird with that in another, or to compare a tumour in the breast with one in the leg. All birds were killed one month after injection unless it had been necessary to do so earlier.

The effect of methylene-blue on the activity of the virus was determined in the hope that the preservative effect of cyanide might be abolished by the dye. But we have found that in the absence of cyanide, and under both anaërobic and aërobic conditions, the activity of the filtrate is rapidly destroyed by 1/10,000 methylene-blue, although no sign of decoloration and reduction could be detected. It is interesting, however, that this inactivation occurs more rapidly in air than anaërobically at 37° C., and we consider that this is due either to a coupled oxidation, or to direct hydrogen transport occurring in the presence of air. Reid (1930) found that hydrogen peroxide was formed during the oxidation of leuco methylene-blue in air. This might account for the greater toxicity of methylene-blue under aërobic conditions.

TABLE II.—*Effect of Methylene Blue.*

Date	No. of bird.	Time of incubation in hours.	Control aërobic.	Meth. blue aërobic.	Control anaërobic.	Meth. blue anaërobic.
1.4.30	456	3	+++	−	..	..
	457	2	−	−	..	..
	458	4	++	+	..	..
	459	4	++	(+)	..	..
18.4.30	470	12 at 0° C.	++++	++++	..	..
	471	12 ,,	++	(+)	..	..
	469	3 at 37° C.	++	(+)	..	..
	468	3 ,,	++++	−	..	..
6.5.30	506	3	+++	+	+++	+++
	507	3	+++	(+)	+++	++
	508	6	++	−	+++	−
	509	6	++	−	+++	−
	510	20	−	−	+++	−
	511	20	−	−	−	−
3.6.30	570	5½	++++	+	++++	++
	571	5½	++++	+	++++	++
3.6.30	572	12	++	−	++++	+
	573	12	++	−	++++	+

Grassmann and Dyckerhoff investigated the effect of cyanide and cysteine on proteolytic enzymes under aërobic conditions, and found their action to be identical. The experiments we have carried out show that this is not the case in the action of cysteine or cyanide on the activity of the Rous sarcoma filtrate.

In this case cyanide preserves aërobically, whereas cysteine only preserves under anaërobic conditions. This might be taken as conclusive evidence that their preservative action here can have no connection with their known effect on proteolysis, but it must be remembered that cysteine is oxidized very rapidly aërobically in the presence of the Rous filtrate, whereas in Grassmann and Dyckerhoff's experiments it is probable that the cysteine autoxidation was much slower. Waldschmidt Leitz (1930) has isolated an activator for proteolytic enzymes from normal and tumour tissues, and has identified it as glutathione. He states that it is only active in the reduced condition. Our evidence that inactivation of the filtrate in air occurs more rapidly if there is an autoxidizable substance present shows that inactivation of the tumour-producing system can occur by oxidation, and makes it probable that cysteine and cyanide owe their preservative power to an inhibition of this process. But on the other hand, it might be that their preservative effect had no relation to their destructive action, and was due to an action on proteolytic enzymes.

In order to decide this question we have attempted to reactivate the virus by reducing systems after it had been inactivated by incubation or hydrogen peroxide. The technique has been to incubate a number of samples of Rous filtrate at 37° C. for different times. After incubation the sample was divided into two parts, one of which was kept in the ice-chest, while the other was incubated for half an hour at 37° C. with a reducing system. Both parts were then injected into the breasts or legs of young chicken in the usual way. The reducers that have been used are the cobalt-cysteine system described by Michaelis (1930), and the hydrogen-palladium reducing system. In the first case cysteine to a final concentration of 1/1000 was added to the filtrate in an anaërobic tube, and one-third of this amount of cobalt sulphate brought to PH 7·2 was placed in the hollow stopper. The tube was then evacuated and inverted, so that the reagents were mixed. Usually half an hour at 37° C. was considered sufficient for reduction to take place. When the hydrogen-palladium system was used, 0·1 c.c. of a stock suspension of palladium black in water was added to the filtrate contained in an anaërobic tube, and after evacuation, H_2, which had been washed with pyrogallol, was let in. The process of evacuation and filling with H_2 was repeated four or five times.

Zinnser (1930) found that herpes virus, in a certain number of cases, if sealed with cysteine and kept at room temperature, regained its activity after one week. Mueller (1928) found some suggestion of this with the Rous virus, but could not obtain clean-cut results. We have not tried the effects of long periods of reduction on the recovery of activity of the Rous filtrate, but we have obtained some evidence of this after the short period of reduction of our experiments.

Table III shows that cobalt and cysteine has been found to be the better of the two reducing systems. Control experiments showed that cobalt alone made no difference to the activity of the filtrate. In some series it was added to the control filtrate immediately before injection. Palladium-hydrogen reduction has given no evidence that activity can be regained, but it is possible with this system to preserve the activity of the Rous filtrate for twenty-four hours at 37° C. (Table IV). It is not known whether the hydrogen-palladium reducing system has any effect on proteolytic enzymes.

TABLE III.—*Effect of Cobalt and Cysteine Reduction.*

Date.	No. of bird.	Time of incubation in hours.	Control.	Reduced.
8.4.30	450	4	++	++++
	451	4	(+)	++
	452	6	—	—
	453	6	(+)	++
	454	24	—	—
	455	24	—	—
30.4.30	486	6	++	+
	487	6	—	—
	488	8	—	—
	489	8	—	—
29.5.30	562	5	++	++++
	563	5	++	++++
	564	$6\frac{1}{2}$	—	++++
	565	$6\frac{1}{2}$	—	—
21.1.31	107	H_2O_2 $\frac{1}{2}$	—	—
	108	H_2O_2 $\frac{1}{2}$	—	++
4.2.31	123	H_2O_2 $\frac{1}{2}$	++	++
	124	H_2O_2 $\frac{1}{2}$	++	++
14.2.31	136	H_2O_2 $\frac{1}{2}$	—	—
	137	H_2O_2 $\frac{1}{2}$	—	++

TABLE IV.—*Preservation of the Activity by Palladium-Hydrogen.*

Date.	No. of bird.	Time of incubation.	H_2 control.	Pd. control.	Pd. H_2.
5.8.30	338	24	..	—	+
29.10.30	10	24	—	..	++
	86	24	..	—	++
	38	24	++	(+)	+++
16.1.31	102	24	—	(+)	++
2.2.31	121	24	—	—	—
	122	24	(+)	—	+++
18.2.31	151	24	—	—	++
	152	24	—	—	++

Inactivation by hydrogen peroxide was carried out by incubating for half an hour at 37° C. a mixture of 0·1 c.c. one volume hydrogen peroxide with 5 c.c. of active Rous filtrate. After incubation, excess peroxide was removed by pumping. Lewis (1928) found that the activity was destroyed by large amounts of hydrogen peroxide, such as we have used. We found, however, that its action was variable, and therefore that it was no more suitable for use as an inactivator than incubation alone.

Since the activity of the filtrate is preserved by cyanide, it is probable that a metal catalysis is concerned in the inactivation. Warburg (1928) has shown that pyrophosphates are similar to cyanides in their inhibitory action on iron

catalyses, but that they have no effect on catalysis by copper. It therefore seemed of interest to determine the effect of pyrophosphates on the activity of the Rous filtrate. Strong concentrations only of pyrophosphate have been tried, but, as Table V shows, preservation is effected for at least twenty-four hours at 37° C.

TABLE V.—*Effect of Pyrophosphate.*

Date.	No. of bird.	Time of incubation.	Control.	$\frac{M}{7}$ $Na_4P_2O_7$.
16.7.30	311	24	(+)	++++
	313	24	+	+++
21.1.31	109	24	+	+++
	110	24	++	++++

The results of these experiments show that the activity of the Rous sarcoma filtrate can be preserved by substances which inhibit oxidations, or create a high reduction potential, but that it is rapidly destroyed by substances which act as oxidation catalysts in air. Also they have shown that reactivation may sometimes be obtained by subjecting the filtrate to a short period of reduction. We conclude from these results that the destruction of the tumour-producing power of the Rous sarcoma filtrate is due to an oxidative, not a proteolytic, change. We have obtained further evidence of this from a study of the proteolytic enzymes of the filtrate. We have found that no peptide, such as leucyl-glycyl-glycine, oxidized glutathione, or chloracetyl tyrosine, is hydrolysed by the filtrate; nor is any single protein such as gelatin, or casein, attacked, either in the presence of cyanide, or alone. Broth and peptone, however, are readily hydrolysed, but no difference is made by the addition of pyrophosphate, cysteine, or cyanide. It was found that during twenty-four hours' incubation of the filtrate the activity of these proteolytic enzymes diminished rapidly. But here again no preservative effect of cysteine or cyanide could be demonstrated.

It is known that many bacteria are unable to multiply under oxidizing conditions, but we do not think that the destruction of the Rous virus is due to a failure in multiplication, since no growth has ever been demonstrated in the absence of living cells. Since the inactivation occurs in the absence of air, the oxidizer must be present in the filtrate. So far we have been unable to separate it from the virus system, and have no idea of its chemical nature. But the results reported in the paper do seem of interest in their bearing on the nature of the tumour-producing system. It seems that since it is not a question of multiplication taking place under certain conditions and not under others, but only a question of survival of virulence, a very definite chemical change may be regarded as destroying the infectivity of the virus. This might be regarded as a suggestion that the infectivity of the virus depended upon one type of molecule alone, perhaps the "specific factor" of Gye's (1925) original theory, or, on the other hand, one might imagine that some necessary constituent of the medium were destroyed. This latter possibility is unlikely, since the same inactivation occurs, and the same protective power of cysteine and cyanide is demonstrated when a filtrate made by absorption of the virus on kaolin, with subsequent elution

by phosphate, and filtration through a Berkefeld candle, is used. Such a filtrate is poor in the total nitrogen, and should contain very few of the constituents of the original filtrate.

SUMMARY.

From a study of the effect of various inhibitors of oxidation and proteolysis on the activity of the Rous filtrate under different conditions, it is concluded that the inactivation of the filtrate which occurs at 37° C. is due to an oxidative change. It is shown that partial reactivation of the filtrate may occur in the presence of reducing agents, and that sodium pyrophosphate and the hydrogen-palladium reducing system preserve the activity of the filtrate for at least twenty-four hours.

We have much pleasure in thanking Dr. Gye for his advice. Both of us are indebted to the Medical Research Council for personal grants.

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THE EFFECT OF THE *X* AND *V* GROWTH-FACTORS ON THE PATHOGENICITY OF INDOLE-PRODUCING STRAINS OF INFLUENZA BACILLI.

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Received for publication March 25th, 1931.

THE literature upon the pathogenic action of cultures of *B. influenzæ* has recently been reviewed by Scott (1929). He states: "Most workers, including Pfeiffer himself, agree that, in fact, the influenza bacillus in pure cultures cannot be made to infect experimental animals. Large doses may be injected into the blood-stream or peritoneum without fatal results, and, in the instances in which death is produced, it is generally found that the bacilli have disappeared."

Pathogenic effects with surface cultures have, however, been described by Malone (1919), Hudson (1922) and others.

In the present work the pathogenic actions of a number of strains of influenza bacilli were tested on mice, particular attention being paid to the relation of indole production to pathogenicity.

INOCULATION EXPERIMENTS.

Experiment 1 *: To determine if there is any difference in pathogenicity between indole-positive and indole-negative strains of influenzæ bacilli.*

Twenty-five unselected indole-positive strains were injected into 25 mice and 25 unselected indole-negative strains into 25 mice. About 50 per cent. of the strains came from the throats of normal people, and the remainder were obtained post-mortem from the trachea of carriers. The second or third sub-cultures were invariably used and the injections were made into the peritoneal cavity. The inoculum consisted of about one-sixth of a Fildes agar slope (8 cm. long in a $\frac{5}{8}$ by 6-in. test-tube). A 12 hours' growth was washed off with ordinary nutrient broth and the bulk of each dose was 0·5 c.c.

Result.

	25 indole + strains.	25 indole − strains.
Died within 3 days	5	0
Ill but recovered	6	1
Perfectly well throughout	14	24

Thus 20 per cent. of the 25 indole-positive strains were capable of killing mice, whereas the indole-negative strains tested were non-pathogenic. The symptoms did not appear until 5 or 6 hours after the injection, and death occurred in 16 to 36 hours.

Post-mortem : Enlargement of the spleen and the glands in the axilla and groin was invariable, with considerable congestion of the blood-vessels of the abdominal and thoracic walls. Influenza bacilli were present in films and cultures from the heart's blood, spleen and glands in the vast majority of cases.

To prove that the fatal results were produced by the bacilli alone, and not by any exotoxic substances in the water of condensation of the Fildes slopes, a few injections were carried out using bacilli which had been washed three times in sterile saline, and then suspended in this fluid for the inoculation. The procedure had no marked effect on the virulence of the organisms and they still remained capable of causing septicæmia.

It was then decided to repeat this experiment on a larger scale, using more than one mouse for each strain, and to run a parallel experiment, at the same time reinforcing the inoculum with a small quantity of the x and v growth-factors in order to give the organism a good start in its new surroundings, and possibly thereby to intensify any pathogenic action.

Experiment 2: To determine if the pathogenicity of the indole-positive and negative strains could be increased by reinforcing the injection of influenza bacilli with a small quantity of the x *and* v *growth factors.*

Ordinary injection.—Twenty unselected indole-positive strains were injected into one hundred mice, and a similar series with indole-negative strains. Five mice were injected with each strain, and the injection consisted of 0·5 c.c. of a broth suspension of one-sixth of a Fildes agar slope.

Result.

	20 indole + strains.	20 indole − strains.
Dead in 3 days . .	23 .	0
Ill but recovered . .	14 .	1
Well throughout . .	63 .	99

Reinforced Injection.—The same 20 indole-positive strains as above were injected into 100 mice, but each injection was reinforced with 0·05 c.c. of Fildes' solution (1920). The indole-negative strains were examined in the same way.

Result.

	20 indole + strains.	20 indole − strains.
Dead in 3 days . .	64 .	3
Ill but recovered . .	22 .	9
Well throughout . .	14 .	88

In order to quote larger numbers I tested the 50 strains used in the first experiment by the reinforced method as well, and so we have for consideration the pathogenicity of 45 indole-positive strains and 45 indole-negative strains when injected by the ordinary and the reinforced methods. The results are as follows:

Strain Mortality by Ordinary Injection.

Of 45 indole + strains 26% killed 1 or more mice.
„ 45 „ − „ 0% „ „

Strain Mortality by Reinforced Injection.

Of 45 indole + strains 71% killed 1 or more mice.
„ 45 „ − „ 6% „ „

This experiment shows that the inclusion of a small quantity of the *x* and *v* growth-factors in the inoculum definitely increases the virulence of influenza bacilli for mice, and accentuates the difference in pathogenicity between indole-positive and negative strains.

CONCLUSIONS.

These experiments indicate that the pathogenic action of a microbe which does not produce any notable quantity of toxic substance *in vitro* can be disclosed

more readily by introducing with it its necessary growth factors. The healthy peritoneal cavity does not contain the necessary factors, and therefore it is not to be expected that influenza bacilli will grow therein. If, however, these factors are supplied either by injury to the tissues as a result of the injection or by including them in the inoculum growth can take place, and toxic substances may then be produced under conditions more favourable to their preservation than in a test-tube.

The application of this principle to the influenza bacillus shows that 71 per cent. of indole-producing strains are pathogenic to mice, whereas those which produce no indole are practically nonpathogenic.

The author is indebted to Prof. W. W. C. Topley and Dr. P. Fildes for criticisms, to Mr. R. Lovell, Dr. H. W. C. Vines and Dr. W. W. Woods for swabs, and to Mr. W. Cole for his invaluable assistance in the experiments.

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THE EFFECT OF SODIUM AUROTHIOSULPHATE IN THE TREATMENT OF TUBERCULOSIS IN GUINEA-PIGS.

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Received for publication April 13th, 1931.

SINCE the publication of Møllgaard's monograph in 1924, the question whether or not sodium aurothiosulphate has any effect on the tuberculosis of small animals has been the subject of a number of papers. Møllgaard (1924) prepared the salt in a pure state by a special method and gave to this preparation the name "sanocrysin." He described experiments on guinea-pigs, from which he concluded that treatment with sanocrysin prevented the development of tuberculosis. Madsen and Mørch (1926) and Cummins (1926) reported favourably on the effect of treatment with sanocrysin of tuberculosis in rabbits,

but Bang (1926, 1927) and Bjorn-Hansen (1927), as well as Calmette, Boquet and Nègre (1926), failed to confirm Madsen and Mørch's experiments ; subsequently in a conjoint investigation by Bang, Madsen and Mørch (1928), designed to clear up the discrepancies between them, no beneficial action of sanocrysin was evident. The Japanese Research Committee on Sanocrysin (1927) were unable to prevent the development of tuberculosis in rabbits, rats and guinea-pigs, though they formed the opinion that the advance of the disease was slightly retarded by sanocrysin treatment.

The present small investigation was undertaken because in previous work the authors (1928) had been able to arrange an experiment with an infecting dose of tubercle bacilli sufficiently critical to show that tuberculosis in guinea-pigs could be prevented, in a certain number of cases, by previous vaccination with B.C.G. It was thought of interest to investigate if, under the same conditions, a preventive effect could also be obtained with such a chemotherapeutic agent as sodium aurothiosulphate.

Guinea-pigs were inoculated by dropping a small amount of a culture of *B. tuberculosis* into the eye—a method which has been employed by Calmette, Cummins and others for infecting guinea-pigs with tuberculosis. With a certain dose of bacilli the method is reliable, and all the animals which do not die prematurely of intercurrent infections eventually die of general tuberculosis. The march of the disease is, however, distinctly slower than when the same inoculum is given subcutaneously or intramuscularly. Moreover, the method seems to have the advantage that every opportunity is given for the normal defences of the body against infection to come into play. The inoculum used in the present experiment was 0·1 mgm. of the bacilli weighed in the wet condition, suspended in 0·01 c.c. of saline, the volumetric measurement being made by means of the micrometer syringe described by Trevan (1925). The strain used (H 12) was a human strain of relatively low virulence to the guinea-pig. The inoculum was found from previous experiments to give rise to generalized tuberculosis in all the guinea-pigs injected, while a tenth of the dose did not uniformly produce tuberculosis in guinea-pigs. The chosen dose was thus approximately minimal. The guinea-pigs used in the experiment were from 365–410 gm. in weight and approximately of the same age.

Injections of sodium aurothiosulphate were given intraperitoneally, and the amount administered appeared to be close to the maximum tolerated dose. Møllgaard gives the maximum intraperitoneal dose for the guinea-pig as 30–40 mgm. per kilo of body-weight, and the doses given in the experiments which will be described approximated to this, though being administered on five successive occasions, care had to be taken to avoid cumulative effects. Twelve guinea-pigs were given 5 mgm. of sodium aurothiosulphate intraperitoneally, and later on the same day these animals and twelve untreated controls were inoculated by dropping 0·1 mgm. of tubercle bacilli on the eye-ball. The twelve treated animals were subsequently given four intraperitoneal doses of 10 mgm. each of sodium aurothiosulphate 4, 11, 20 and 24 days after their inoculation with tubercle bacilli. The untreated controls were kept in separate cages, but lived otherwise under similar conditions to the treated animals. All the animals of both groups were allowed to die naturally, and were then carefully examined for tuberculous lesions.

Five of the treated and two of the untreated animals died before obvious tuberculosis had time to develop. The abnormal death-rate in the treated animals during the period of treatment with sodium aurothiosulphate suggests that maximum doses of the salt were being employed. The remaining guinea-pigs treated with sodium aurothiosulphate died at the following intervals (given in weeks after inoculation with tubercle bacilli): 8, 9·5, 29·5, 17, 18, 17 and 21. The remaining control animals died at intervals of 17, 9, 17, 17, 26·5, 13, 14·5, 15·5, 18 and 9·5 weeks. The guinea-pigs of both groups all died of generalized tuberculosis and no distinction could be made in the lesions observed. The mean survival time for the guinea-pigs treated with sodium aurothiosulphate was thus 17·1 weeks, and that for the untreated controls 15·7 weeks. It need hardly be pointed out that such a difference does not approach significance when tested by the formula of Fisher (1930).

It must be admitted that the arrest or cure of tuberculosis in an animal as susceptible to the infection as the guinea-pig is a stringent test to which to submit an alleged remedy against tuberculosis. In these experiments an attempt was made to allow an inoculum approaching the minimal infecting dose to develop in an animal as far as possible "saturated" with sodium aurothiosulphate during the earlier stages of the infection; the result was such as to suggest strongly that sodium aurothiosulphate possesses no specific bactericidal action against the tubercle bacillus.

The preparation we used was made in the Experimental Laboratories of Messrs. Burroughs Wellcome & Co. [On analysis, gold found was 37·2% and 37·26%. $Na_3Au(S_2O_3)_2$, $2H_2O$ corresponds to Au 37·5%.]

CONCLUSION.

Tuberculosis in guinea-pigs could not be influenced by treatment with sodium aurothiosulphate.

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THE BLOOD GLUTATHIONE IN DISEASE.

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Received for publication April 26th, 1931.

It is now well known that glutathione is present in practically all the tissues of the body, including the red blood-cells. Owing to its occurrence in a reduced form containing a sulphydryl group, and an oxidized form in which two molecules combine together through their sulphur atoms with the loss of two atoms of hydrogen, glutathione probably assists in many important oxidations and reductions in the body. The object of this paper is to record the results of an investigation into the glutathione content of the blood in man under various pathological conditions, the blood being the only human tissue readily accessible for chemical examination.

It is known that glutathione occurs, not in the plasma, but in the red blood-corpuscles, and according to Gabbe (1929) its purpose there is not so much for the metabolic needs of these cells, but rather for assisting in the oxidation and reduction of hæmoglobin. The same author has also demonstrated that the blood glutathione is low in anæmia, as might be expected, but the amount contained in each corpuscle is actually greater than normal. This appears to be a compensatory mechanism, and Gabbe also showed that after the removal of large quantities of blood from animals, though the total blood glutathione was reduced, the amount per cell was increased twofold within two hours of the hæmorrhage. The gradual production of anæmia by phenyl hydrazine was followed by an even greater increase in the glutathione of the cells, so that the total glutathione actually rose. Gabbe also found both in animal and human experiments that if the subject was made to breathe an atmosphere of low oxygen tension the blood glutathione increased, and demonstrated that it was above normal in cases of cardiac or asthmatic dyspnœa and in polycythæmia.

A few other workers have estimated the blood glutathione in various clinical conditions. Campanacci (1930) finds slightly low values in diabetes; Varela, Apolo and Vilar (1930) confirm this, and the low values in anæmia and their results in jaundice were again on the low side, especially in obstructive cases. They found a high glutathione content in a case of syphilitic aortitis, but normal values in hypertension, asthma, carcinoma, pregnancy, and in a case of lymphatic leukæmia with a white count of 300,000 cells per c.mm. (see later). King, Baumgartner and Page (1930) report normal findings in mental cases.

METHOD.

In venous blood the glutathione is present entirely, or almost entirely, in the reduced form, and can be estimated in protein-free filtrates by titration

with iodine. In this series of experiments starch has been used to indicate the end-point, though controlled by the nitro-prusside reaction (as an external indicator), wherever it seemed necessary, and whenever there was sufficient material to carry out several series of titrations. With iodine-starch titration with the addition of an extra quantity of potassium iodide as suggested by Perlzweig and Delrue (1927) the end-point is quite sharp, and duplicates can be obtained with an error of less than 3 per cent. Back-titration with thiosulphate was therefore not employed, although it was used for some control experiments at the commencement of the work and found to give equivalent results.

The following standardized technique has been used throughout, and though small quantities of other substances may be included in the values expressed as glutathione, the method is sufficiently specific for clinical purposes, and yields satisfactory results with small amounts of material.

To 5 c.c. of well-mixed oxalated blood, add as soon as possible after removal 10 c.c. distilled water and (directly the corpuscles are laked) 10 c.c. 20 per cent. trichloracetic acid. Mix and filter. N/1000 iodine solution is prepared daily by adding 2 c.c. N/10 iodine and 2 c.c. 25 per cent. potassium iodide to about 100 c.c. distilled water and making up to 200 c.c. To 10 c.c. of the blood filtrate (not further diluted) 2 drops of 2 per cent. soluble starch (freshly prepared each week) is added, and the iodine solution run in from a burette delivering small drops and graduated in twentieths of a c.c. The number of c.c. iodine used multiplied by 15·35 gives the amount of glutathione in mgm. per 100 c.c. original blood.

Owing to the fact that glutathione was until recently considered to be a dipeptide with a molecular weight of 250 instead of a tripeptide of molecular weight 307 (Hopkins, 1929), and also on account of the various methods employed for estimation, the actual figures in this paper will not always correspond with those of other authors.

RESULTS.

The blood glutathione in twenty observations on twelve normal persons varied from 30·7 to 47·3 mgm. per 100 c.c. the average figure being 39·4. Probably figures from 30 to 50 mgm. may be taken as being within normal limits for non-anæmic persons. Gabbe (1929) states that he found values of from 24 to 46 mgm., which, corrected for the new formula assigned to glutathione, would be 29·5 to 56·6. He also mentions that meals have little effect on the blood glutathione, though exercise increases it. The former statement was confirmed in these observations. Many estimations were done on blood-plasma, and results varying from 2 to 5 mgm. per 100 c.c. were frequently obtained in plasmas apparently free from the products of hæmolysis. The subjection of filtrates to powerful reduction (with acid and zinc) did not increase the glutathione content significantly, so that apparently the substance in these filtrates occurs purely in the reduced form.

A series of 44 pathological cases of various types was studied, and for the most part it was found that the blood glutathione showed little variation in widely differing conditions. Thus normal values were obtained in nephritis

of various types, with or without uræmia (provided there was no anæmia), and in a case of nephrosis (blood cholesterol 0·6 per cent.). Cases of obesity, gall-stones, diabetes, gout and pulmonary tuberculosis also gave normal results. The opportunity of studying a patient with cystinuria was the original cause of this investigation, as it was thought possible that some disturbance of glutathione might here be found. Our hopes were not realized, however, for filtrates of his blood gave the same normal value (34 mgm.) both before and after reduction with acid and zinc. The relation of glutathione to tissue oxidation suggested that interesting results might be obtained in cases of dyspnœa and cyanosis, but it was found that in general the blood glutathione was within normal limits so long as there was no marked polycythæmia. Thus cases of pneumonia with slight cyanosis, asthmatic bronchitis, cardiac failure and early pulmonary fibrosis gave normal results, as did also a case of congenital heart disease with cyanosis on exertion, and a red blood-count of 5,140,000 per c.mm. A case of pulmonary arterio-sclerosis with a red count of 5,200,000 showed a high figure (64·4 mgm. per 100 c.c.), which increased to 72·3 mgm. as right heart failure progressed and the red count rose to 6,640,000. The patient was deeply cyanosed, and the glutathione content is increased out of proportion to the red count.

A case of myxœdema gave a normal value, whereas two cases of exophthalmic goitre showed slightly low results (26·5 and 29·2 mgm.), although not anæmic.

Apart from the blood diseases to be described later, cases of jaundice and liver disease showed the widest variations, which were difficult to correlate with the clinical findings. Thus a case of suppurative cholangitis with an extraordinarily high degree of jaundice (55 Van den Bergh units) showed a normal blood glutathione, whereas a severe toxic jaundice (probably due to arsenic) showed a high reading (55·2 mgm.), which returned to normal (41·8 mgm.) the next day, coincidently with a sudden change for the better on the part of the patient, who eventually recovered. In a case of mild catarrhal jaundice the blood glutathione was normal, whereas in a case of obstructive jaundice a slightly low reading was obtained (29·4 mgm.), as also in three cases of cirrhosis of the liver (29·2, 28·4 and 27·6 mgm.) without anæmia. A case of carcinoma of the liver showed a low glutathione content (21·8 mgm.), even allowing for the anæmia (3,860,000 red cells per c.mm.)

In the group of blood diseases a case of acholuric jaundice and one of splenic anæmia confirmed the observations of Gabbe and others, already mentioned, that in severe anæmias the glutathione content of the blood is not reduced in proportion to the red cell deficit. Counts of 1,800,000 and 3,220,000 gave glutathione values of 21·8 and 35·4 mgm. respectively, which, corrected to a theoretical 5,000,000 red cells, would read 60·6 and 55·0. Three cases of primary polycythæmia, on the other hand, showed a very high glutathione content on account of the increased blood-count, and in a severe case with 10,480,000 red cells per c.mm. and much cyanosis (glutathione 109 mgm. per 100 c.c.), this increase seemed to be out of proportion to the red count, perhaps again an example of a compensatory phenomenon.

The most interesting results were obtained in cases of leukæmia, especially

as there is, as far as the author is aware, no record of the presence or absence of glutathione in the white blood-corpuscles. In a patient with myelogenous leukæmia the blood showed 576,000 white cells per c.mm. (polymorphs 38 per cent., other granular cells 60 per cent.) and 2,900,000 red cells. The glutathione, despite the anæmia, was 82·5 mgm. per 100 c.c., and was obviously largely contained in the white cells. In order to be certain, the blood was separated by centrifuging into the three layers plasma, white cells, red cells, and these were pipetted off as selectively as possible. The result of the glutathione titrations (controlled in this case also by the nitro-prusside reaction) was plasma 5 mgm., white cells 151 mgm., red cells 72 mgm. (per 100 c.c. in each case). The same case, after a month's X-ray treatment, white cells now 54,000, red cells 2,400,000, showed a blood glutathione of only 30 mgm. per 100 c.c. In another case of myelogenous leukæmia after X-ray treatment (white cells 32,000, red cells 5,200,000) the blood glutathione was 51 mgm. per 100 c.c. A case of lymphatic leukæmia, on the other hand, gave a doubtful increase in glutathione when it is remembered that in anæmia there is a proportional increase in the glutathione content of the red cells. The figures were—red cells, 2,880,000, white cells 292,000, glutathione 42·7 mgm. per 100 c.c. It was thought possible that lymphocytes might contain little or no glutathione, and that this might be related in some way to the peroxidase reaction, which gave characteristic results (positive or negative) in all the leukæmic cases recorded here. However, a further case of lymphatic leukæmia of fairly acute type showed that the lymphocytes contain large quantities of glutathione, though not so much as in the granular series. Details of this case were as follows : April 13th, 1931, red cells 1,730,000 per c.mm., white cells 200,000 per c.mm., glutathione 19·6 mgm. per 100 c.c. April 19th, 1931, red cells 880,000, white cells 127,000. A definite layer of white cells sufficient for the purpose of glutathione estimation formed on centrifuging 10 c.c. of blood, and differential estimations gave the following results : plasma 6 mgm. per 100 c.c., white cells 67 mgm., red cells 71 mgm The filtrate from the white cell layer also gave a positive nitro-prusside. reaction, and the differential count showed 98 per cent. of lymphocytes, the great majority of which were of large size.

The results obtained suggested a determination on a specimen of thick (staphylococcal) pus, which showed a glutathione content of 164 mgm. per 100 c.c. Experiments are in progress to determine whether the method of iodine titration will have any practical value as an indication of the amount of pus in infected urines and other fluids.

SUMMARY AND CONCLUSIONS.

1. Observations on the glutathione content of human blood, as determined by iodine titration, show that the normal amount of glutathione present is about 30 to 50 mgm. per 100 c.c., and that this is present almost exclusively in the corpuscles.

2. In the majority of pathological conditions, including cystinuria, the blood glutathione remains within normal limits, though cases of liver disease show rather wide variations.

3. In anæmia, and some cyanotic states, the ratio of glutathione to corpuscles is increased.

4. Studies of myelogenous and lymphatic leukæmia show that glutathione is a constituent of both types of white corpuscles. A large amount of glutathione is also contained in pus-cells.

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THE PREPARATION AND STANDARDIZATION OF COLLOIDAL GOLD FOR THE LANGE TEST.

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Received for publication April 29th, 1931.

ALTHOUGH a wide choice of means for the preparation of colloidal gold suitable for the Lange test is available, all methods without exception require a great deal of patience and perseverance before they can be made to furnish a consistent product. Even when considerable experience enables one to gauge the conditions and reagents so as to yield a more or less successful sol, the preparation is always liable to go astray despite the most careful handling.

The method which is about to be described here is one which has obvious advantages over existing methods, in that while its steps are the simplest and its reagents readily available, it yields gold sols with a precision and certainty unequalled by any of the usual methods, including that of Mellanby and Anwyl-Davies (1923), which it follows in using oxalate reduction.

The steps in the method are :

(1) The preliminary testing for the suitability and adjustment of reagents.

(2) The preparation in bulk of the stock gold sol on the basis of the preliminary test.

(3) The final adjustment of the sol to the correct stability for immediate use in the Lange test. This is done by the addition of small amounts of acid, using the stability of the sol against oxyhæmoglobin as indicator.

THE PREPARATION OF THE GOLD SOL.

Reagents:

(1) Distilled water.
(2) Potassium oxalate (of analytical purity) 1 per cent. solution.
(3) Gold-sodium chloride (double salt), $AuCl_3$, NaCl, $2H_2O$.
(4) N/50 sodium hydroxide.
(5) N/50 hydrochloric acid.

Notes on reagents.—The water is prepared by distilling ordinary tap or distilled water through an all-glass (hard or resistance glass) apparatus, rejecting the first 5 per cent., leaving a similar amount as residue, and collecting the mid-fraction for use. Although this glass-distilled water has been used in the standard preparation, a gold sol nearly equal in quality results from the substitution of the ordinary laboratory distilled water furnished by the usual type of large copper still.

The oxalate is dissolved in ordinary distilled water, and is used as long as it is perfectly clear. In time a haze or slight precipitate develops, when a fresh solution is made up. For the gold chloride the sodium double salt has been used as the standard reagent, but the acid salt functions equally well, the necessary adjustment corresponding to the change of gold being made apparent in the preliminary test.

The alkali is the ordinary N/50 standard solution prepared from a stock CO_2-free sodium hydroxide, and preserved in a hard-glass bottle.

The Method.

Preliminary test.—To 50 c.c. of the distilled water 0·5 c.c. of 1 per cent. potassium oxalate and 0·5 c.c. of gold chloride solution are added. A row of test-tubes is set up and 5 c.c. of the mixture placed in each. The first of the series being left as made up, 1 drop of N/50 alkali is added to the second, 2 drops to the third, and so on to the sixth, a standard dropping pipette being employed (one giving 25 drops to the c.c.). The whole series of tubes is then immediately immersed in about 70 c.c. of water at air temperature contained in a 250 c.c. hard-glass beaker. The temperature of the water-bath is then rapidly raised, heating being by means of a strong bunsen burner under a thin plain iron wire gauze (*i. e.* without an asbestos centre). When the bath has reached boiling-point and remained there for about a minute, the several tubes are withdrawn and placed in order in a rack. Of these tubes only one is representative of the right conditions for the preparation of the stock gold sol. It is the lowest in the series to give a bright red clear sol which, when carefully examined in a good light, exhibits just the faintest sheen. Those containing less alkali eliminate themselves as exhibiting a very marked sheen, while those containing more alkali than the selected tube are too clear and purple, the total colour development being also less intense. In general, when the sodium gold chloride double salt has been used, tube 4 is representative of the correct conditions. It is equivalent to the use of 1 c.c. of gold chloride (1 per cent. solution), 1 c.c. of 1 per cent. potassium oxalate and 2·4 c.c. of N/50 sodium hydroxide with each 100 c.c. of distilled water.

The way is now open to the preparation of the gold sol itself in bulk.

Actual preparation of the stock gold sol.—To 200 c.c. of distilled water contained in a hard-glass beaker (just previously thoroughly treated with aqua regia, and washed with successive quantities of tap-water and distilled water) 2 c.c. of 1 per cent. potassium oxalate, 2 c.c. of 1 per cent. gold chloride and 4·8 c.c. (or such amount as has been ascertained in the preliminary test) of N/50 alkali are added. The whole is raised rapidly to the boiling-point. The initial change in the formation of the hydrosol is indicated by the appearance of a light blue colour, which soon darkens, and then as the temperature is further raised, the solution, still remaining clear, becomes more and more purple, then red, until just before the boiling-point is reached a rapid final change to a light cherry-red sets in, the sol then remaining perfectly stable in colour after the boiling-point is attained. Once this final stage is reached the heat is withdrawn, the beaker covered and allowed to cool. When sufficiently cool it is transferred to a glass-stoppered stock bottle.

Titration of stock gold sol.—A few drops of blood are taken directly from the finger into about 10 c.c. of normal saline, the red corpuscles spun down, and after the supernatant liquid is removed, are washed twice with 10 c.c. of saline. A 1 in 100 dilution of the corpuscles is made in water, and is then used upon the gold sol exactly as a specimen of cerebro-spinal fluid is used in the Lange test (*i.e.* with 2·5 c.c. of gold sol to each tube). The test is made against four different specimens of gold sol acidified as follows :

Specimen.	Amount of stock sol.	Volume of acid added.
1	20 c.c.	0·35 c.c. N/50 HCl.
2	20 ,,	0·40 ,, ,,
3	20 ,,	0·45 ,, ,,
4	20 ,,	0·50 ,, ,,

Readings are made after 24 hours.

With the smallest of acid it is usually found that the first five tubes of the Lange series are not affected, whereas with the greatest amount of acid all five will show complete precipitation. The correct addition of acid is the smallest quantity that will just enable the gold to be partially precipitated by the oxyhæmoglobin, the tubes 1 to 5, to which attention is confined, showing a pale pink colour. (The later tubes of the series, particularly 6 and 7, show precipitation at this stage, and even with slightly lower additions of acid, but this precipitation is of a different type to that just mentioned—the obvious difference is that it leads to a bluish-purple colour instead of a pink—and need not be taken into consideration for the purpose of defining the end-point of the titration.) In effect this is really a titration to pH 6·7–6·8, the isoelectric point of oxyhæmoglobin, which pH refers, however, not to the gold sol itself, but to the mixture when all the reacting substances are together.

With very little experience it is possible to ascertain the correct acid addition by noting the appearance of the tubes after 10 minutes, without waiting for the final reading at 24 hours, and also to cut down the number of specimens of acidified gold to be tested to two. It is essential to use a freshly prepared solution of oxyhæmoglobin for the titration.

Immediately before use the stock gold sol is acidified in accordance with the above titration, and is then completely standardized, fulfilling all the requirements of the Lange reaction.

FURTHER OBSERVATIONS.

The advantages of the method can now be stated in more precise terms. In the first place, in that the reaction is started with all the reagents together in the cold, no uncertainty arises as to the precise manner in which the reagents are to be added. In other words, the conditions are capable of exact definition. Further, the change of reaction is gradual throughout, instead of being dramatic and sudden as when the process is conducted at or near the boiling-point, and this tends to keep the preparation under better control. Not only does this method of carrying out the reaction enable one to institute a very simple preliminary test ensuring the success of the bulk preparation, but the process itself is relatively immune from the disturbing influences that are usually connected with minute traces of impurity in the reagents and distilled water.

Finally, the adjustment against oxyhæmoglobin is of much greater practical value than either of the two chief methods of correction hitherto suggested. It employs an easily accessible protein standard under conditions which reproduce exactly the conditions of the Lange test. Since the Lange response is due to the protein constituents of the cerebro-spinal fluid and is governed by their iso-electric points (this statement is based upon further experimental work, the results of which will be published in a subsequent paper), the conditions of standardization are entirely comparable with those of the actual test. On the other hand, the so-called " titration with saline," which attempts to ascertain the correct stability by examining the sensitivity of the sol against 1 per cent. saline (Novick, 1928), bears no direct relation to the conditions of the Lange test. The other method of adjustment, that of employing an indicator to bring the gold sol to a definite pH, is one which has serious practical difficulties in view of the high colour of the sol itself.

It might be also pointed out that the method of final adjustment presented here is equally applicable to any of the various methods which have been devised to produce gold sols for the Lange test.

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THE EFFECT OF X-RAY THERAPY ON THE PARTITION OF PHOSPHORUS COMPOUNDS IN THE BLOOD IN DISEASE.*

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Received for publication March 24th, 1931.

BUCKMAN and his co-workers (1925) studied the blood-phosphorus content in chronic myelogenous leukæmia, especially as influenced by the action of X-ray therapy. They found that a marked increase in the total phosphorus content both of the blood-plasma and of the red blood-corpuscles generally occurred. They, however, only determined the total and the inorganic phosphorus content of the whole blood, blood-plasma and red blood-corpuscles, and did not attempt to find out which phosphorus compound was responsible for the changes noted. In the investigation on which the present communication is based an attempt was made to study the effect of X-ray irradiation on the different phosphorus compounds of the whole blood, blood-plasma and the red blood-corpuscles.

METHOD AND PROCEDURE.

The method used for all the estimations of the phosphorus compounds of the blood was that described by Kay and Byrom (1927) with one exception: oxalate was used as an anti-coagulant throughout, whereas Kay and Byrom employed oxalate for the sample of blood for analysis, and the substance "heparin" when determining the hæmatocrit values.

The procedure adopted here was also identical for all the experiments. First a specimen of blood was obtained from a vein in the arm after 12 hours' starvation, and all the estimations of the phosphorus compounds were carried out immediately. Then the patient received the X-ray treatment, and samples of blood were again taken at different intervals after the irradiation, but under similar precautions, as stated above.

DISCUSSION.

These results support the findings of Buckman and his colleagues (1925) for the blood-plasma, the phosphorus content of which was reported to be increased, but they fail to confirm the reported increase in the total phosphorus content of the red blood-corpuscles. The total phosphorus content of the corpuscles showed a tendency to diminish in amount per cent. after the X-ray

* These results have been submitted to the University of Edinburgh in accordance with the regulations for the degree of M.D.

TABLE I.

No.	Name.	Age.	Diagnosis.	Time when blood examined.	Whole blood. Mgm. per 100 c.c.						Plasma. Mgm. per 100 c.c.					Corpuscles. Mgm. per 100 c.c.				Hæmatocrit R.B.C.'s %.
					F.P.	F.E.P.	E.P.	L.P.	T.P.(C.).	T.P.(A.).	F.P.	F.E.P.	E.P.	L.P.	T.P.	E.P.	L.P.	E.P.	T.P.	
1	H. M—	8	Lymphadenoma	Before X-ray therapy	4·06	23·89	19·83	11·74	36·13	35·63	4·20	4·56	0·36	6·08	10·69	3·79	22·6	57·10	83·49	34·30
				5 days after ditto	3·98	25·47	21·49	10·75	36·63	36·22	4·11	4·52	0·41	7·15	11·76	3·73	17·50	60·90	82·13	34·80
2	,,	8	,,	Before X-ray therapy	3·84	23·00	19·16	12·47	36·20	35·47	3·90	4·25	0·35	8·95	13·12	3·70	21·00	64·90	89·60	29·10
				24 hours after ditto	3·95	25·00	21·05	13·30	38·10	38·30	3·80	4·30	0·5	10·70	15·00	4·23	18·00	58·30	80·50	35·50
				5 days ,, ,,	4·09	24·80	20·71	12·80	37·80	37·60	3·90	4·35	0·45	10·85	15·20	4·51	16·82	62·40	83·70	32·70
3	C. M—	7	Hæmangioma	Before X-ray therapy	4·08	27·10	23·02	12·80	40·60	39·90	4·15	4·48	0·33	8·72	13·20	3·90	20·10	63·80	87·80	35·70
				33 hours after ditto	3·54	25·00	21·46	11·1	35·60	36·10	3·85	4·30	0·45	9·4	13·7	2·90	14·54	62·70	80·14	33·70
4	E. McA—	60	Carcinoma	Before X-ray therapy	3·01	30·80	27·79	9·00	40·60	39·80	2·90	3·10	0·20	5·00	8·06	3·40	15·20	70·50	89·10	39·40
				46 hours after ditto	2·99	30·40	27·41	9·30	40·10	39·70	2·40	2·80	0·40	7·20	10·00	3·90	12·50	68·60	85·00	39·60
5	J. B—	33	Cerebral cyst	Before X-rays therapy.	2·63	19·60	16·97	12·20	30·90	31·80	2·30	2·60	0·30	7·20	9·80	3·30	22·20	50·30	75·80	33·30
				3 days after ditto	2·85	19·20	16·35	11·80	31·40	31·00	2·70	3·30	0·60	8·70	12·00	3·10	17·50	45·30	65·90	35·20

F.P.=Inorganic or free phosphorus; F.E.P.=free plus ester phosphorus; E.P.=ester phosphorus; L.P.=lipin phosphorus; T.P.(c) and T.P.(A.)=Total phosphorus by combustion and addition respectively.

therapy. This difference, however, might be accounted for by the fact that the pathological condition (myelogenous leukæmia) for which they administered X-rays was different in nature to the conditions investigated by the author.

From the available results there can be little doubt as to the effect of X-ray therapy upon the partition of the phosphorus compounds of the blood. The X-rays apparently must cause a certain definite reaction in the tissues of the body which markedly and directly affects the distribution of the phosphorus compounds in the blood. These changes in the partition of phosphorus probably set in immediately after the administration of the X-rays, for they were evident twenty-four hours after the irradiation, and they might have been present earlier had they been sought for. Furthermore, they showed a tendency to persist, for they were still present in the blood five days after treatment. It would probably have been of some importance to have followed the cases for a longer period in order to see how long the changes persisted, but this was in the circumstances not possible.

Inorganic and free phosphorus.—In five experiments carried out on four patients suffering from different pathological conditions, the free phosphorus fraction of the blood-plasma and of the red blood-corpuscles was found either increased or decreased in appreciable amount per cent., but there was no uniformity in these changes. Thus the free phosphorus of the blood-plasma showed a tendency to fall in three experiments, to remain constant in one and to increase in one. Similarly, the free phosphorus of the corpuscles was decreased in three experiments and increased in two.

Ester phosphorus compounds.—The X-ray therapy caused very marked changes in the ester phosphorus of the blood-plasma. The amount of ester phosphorus of the plasma tended to rise, amounting in two cases to 100 per cent. above the normal values.

The ester phosphorus of the red blood-corpuscles was also affected by the action of the X-rays. Thus in four out of five experiments it showed a tendency to fall in amount per cent. suggesting that the corpuscles were actually poorer per unit volume.

Lipin phosphorus compounds.—The most striking change, however, took place in the lipin phosphorus content of the blood as a result of the X-ray therapy. This change was found uniformly present in all the experiments. The lipin phosphorus of the blood-plasma was constantly and markedly increased in amount per cent. after the irradiation. On the other hand, the lipin phosphorus content of the red blood-corpuscles showed a definite tendency to be invariably decreased in amount per cent.

In the light of our present knowledge and the available data it is difficult to offer any satisfactory theory which would explain these changes in the partition of phosphorus compounds of the blood following upon the administration of X-ray therapy, but they are sufficiently marked to demand further investigation.

SUMMARY.

1. The administration of X-rays does influence and cause changes in the partition of the phosphorus compounds of the blood.

2. The free phosphorus of the blood-plasma and of the red blood-corpuscles does undergo a change, but it is not uniform.

3. The ester phosphorus of the plasma shows a tendency to rise, and the ester phosphorus of the corpuscles to fall slightly in amount per cent.

4. The lipin phosphorus of the blood-plasma shows a tendency to increase markedly, and the lipin phosphorus of the red blood-corpuscles to decrease markedly in percentage.

In conclusion, it gives me great pleasure to express my gratitude to Prof. Leonard Findlay for enabling this work to be carried out and for his inspiring guidance and criticism throughout. Further, I am greatly indebted to Dr. Noah Morris for his helpful advice and encouragement, to Dr. S. D. Scott, Assistant Radiologist to Western Infirmary, Glasgow, and to Mr. Matthew White, Surgeon, Royal Hospital for Sick Children, Glasgow, for their courtesy and permission to study their cases. I am also indebted to Dr. D. C. Suttie, Radiologist at the Children's Hospital, for his invaluable co-operation.

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A CONTRIBUTION ON THE DISTRIBUTION OF CALCIUM CONTENT IN BLOOD OF CHILDREN.*

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Received for publication March 24th, 1931.

ABUNDANT data are available in the literature concerning the calcium content of the blood-serum and the red blood-corpuscles. Nevertheless, the presence of calcium in the red blood-corpuscles has often been a subject of considerable discussion. Hamburger (1909) reported as much as 32 mgm. of calcium per cent. of the red blood-corpuscles. Cowie and Colhoun (1919) also found calcium in the red blood-corpuscles, but the average quantity was only 4·26 mgm. per cent. for unwashed and 3·47 mgm. per cent. for washed human

* These results have been submitted to the University of Edinburgh in conformation with the regulations for the degree of M.D.

red blood-corpuscles. Jones and Nye (1921) also insist on the presence of calcium in the corpuscles in appreciable amount, their average figure being 8·7 mgm. per cent., and likewise Jones (1921), Anderson (1924) and Stanford and Wheatley (1925) claim that calcium is present in human red blood-corpuscles.

On the other hand, as early as 1898, Abderhalden (1898), in a study of the substances of the blood, could not detect any trace of calcium in the red blood-corpuscles. Richter-Quittner (1921), Falta (1921), and very recently Leiboff (1930), also failed to find calcium present in either human or animal red blood-corpuscles, while Kramer and Tisdall (1922) and Stewart and Percival (1928) report that the calcium content of the corpuscles of human blood is minimal.

METHODS.

The method described by Kramer and Tisdall (1921) was used for the determination of the calcium content of the blood-serum, and that described by Anderson (1924) for the determination of the calcium content of the whole blood. Hæmatocrit readings were taken from the oxalated blood spun in a centrifuge for about 45 minutes at a rate of 3000 revolutions per minute.

The results for calcium content of the whole blood, the blood-serum and for the red blood-corpuscles of 5 normal children and 2 children suffering from tetany with rickets are given in Table I. The figures for red blood-corpuscles are derived from the figures for blood-serum and whole blood by means of calculation.

TABLE I.—*Distribution of Calcium Content in the Blood.*

No.	Name.	Condition.	Calcium in mgm. % blood-serum.	Calcium in mgm. % whole blood.	Calcium in mgm. % corpuscles.
1	C. G—	Healthy	10·2	6·82	0·3
2	J. M—	,,	9·72	6·99	0·31
3	E. C—	,,	10·9	7·52	..
4	A. M—	,,	11·1	7·1	0·27
5	A. G—	,,	11·4	6·99	0·05
6	J. L—	Tetany	7·1	5·56	1·34
7	G. T—	,,	5·2	3·7	1·3

DISCUSSION.

The results show that the red blood-corpuscles of the five normal children were practically devoid of all calcium, but that the corpuscles of the two cases with tetany contained an appreciable amount, suggesting that there was some shifting of the calcium from the blood-serum into the corpuscles in this disease. As all previous workers appear to have studied the distribution of calcium in normal blood only, this observation raises the possibility of some biochemical change taking place in the condition of infantile tetany, which causes the transference of a certain percentage of the calcium content of the serum into the red blood-corpuscles.

SUMMARY.

1. The red blood-corpuscles of normal blood contain no appreciable amount of calcium.

2. The red blood-corpuscles of the blood in two cases of tetany contained an appreciable amount of calcium.

In conclusion I wish to acknowledge my great indebtedness to Prof. Leonard Findlay for enabling me to carry out this investigation and also to Dr. Noah Morris for his helpful advice on biochemical matters.

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THE GROWTH OF *BR. ABORTUS* (BOVINE TYPE) IN SHAKE TUBES.

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Received for publication April 30th, 1931.

IN his original description of *Br. abortus*, Bang (1897) reported the observations made by his assistant Stribolt on the gaseous requirements of this organism. Briefly it was found that *abortus* would not grow on the surface of a serum agar gelatin medium under aërobic conditions, but that if it was inoculated into a shake tube of the same medium it gave rise to a band of growth

situated 5–15 mm. below the surface. Removal of the CO_2 over the surface of the shake, by soaking the cotton-wool plug of the tube in sodium hydrate solution, did not interfere with the development of the band. If the air over the shake was replaced by approximately 90 per cent. oxygen, growth not infrequently occurred in two distinct zones, an upper one, situated at a depth of 3–13 mm., and a lower one, at about 30–40 mm. From these observations the conclusions were drawn that CO_2 was not necessary for growth, and that there existed two optimum partial pressures of oxygen, one corresponding to that of more or less pure oxygen, the other to a pressure less than that normally present in the atmosphere.

These conclusions, which influenced and misled the majority of subsequent workers, are, it is believed, entirely fallacious, and it is the purpose of this paper to offer a different interpretation of Stribolt's original findings.

THE ZONE PHENOMENON IN SHAKE AGAR TUBES INCUBATED AËROBICALLY.

Preliminary Observations.

Throughout this work strains of the bovine *abortus* type were used which would not grow aërobically on the surface of agar unless a certain proportion of the air over the medium, preferably 5–10 per cent., was replaced by CO_2. The shake tubes employed contained either 8 c.c. in $\frac{5}{8}$-in. tubes, or 12–16 c.c. in $\frac{3}{4}$-in. tubes, of heart extract agar, usually pH 7·2, and were inoculated from below upwards by means of a pipette, with 0·1–0·2 c.c. of a broth suspension of *Br. abortus*. This was prepared by washing off a two-day agar slope culture in 5 per cent. CO_2 with 2–4 c.c. of broth. In a tube seeded in this way, and incubated aërobically, a faint band of growth situated 2–4 mm. below the surface becomes apparent in about 3 days. The exact position and thickness of the band, together with the degree of growth, are determined by a number of factors, such as the reaction of the medium and the size of the inoculum, which it is unnecessary to consider at the moment.

In attempting to explain the cause of this zone phenomenon, it was clearly necessary to learn something of the gaseous conditions prevailing at the site of growth. Since no simple method of obtaining this information presented itself, experiments were carried out to determine the effect of changes in the atmospheric environment on the growth of *abortus* on the surface of agar slopes. These experiments have already been reported (Wilson, 1931). It was found that for growth to occur both oxygen and carbon dioxide were essential; that the optimum partial pressure of oxygen was about 20 per cent., but that growth would occur between limits of 0·5 and 99 per cent.; that the optimum partial pressure of CO_2 was 5–10 per cent., but that growth would occur between limits of 0·5 and 98 per cent.; and that a lowered partial pressure of oxygen was in no way conducive to growth. If the gaseous conditions controlling growth in shake cultures are similar to those in surface cultures—and there is no reason to suppose that they are different—it follows that at the site of the band in which the organisms develop both oxygen and carbon dioxide must be present. With this assumption as a working hypothesis, experiments were devised to prove the existence of both these gases at the site of growth.

The Penetration of Oxygen into Agar Shake Tubes.

Methylene-blue was added to a shake agar tube so as to give a concentration of about 1/200,000. The tube was boiled till the dye was decolorized, immersed in cold water until the agar had set, and then incubated aërobically at 37° C. Similar tubes containing methylene-blue were inoculated, after boiling, with *Br. abortus*, the size of the inoculum being varied. The results are given in Table I. It is apparent that in the uninoculated tube the oxygen pressure

TABLE I.—*Penetration of Oxygen into Agar Shake Tubes Incubated Aërobically Figures indicate distance below surface in millimetres.*

Tube.		Result.
1. Agar containing methylene-blue .	Blue 0–26	1 day.
	,, 0–33	4 days.
	,, 0–38	9 ,,
2. Ditto, but inoculated with large number of organisms .	Blue 0–12.	No growth 1 day.
	,, 0–7.	Slight growth 0·5–3, 4 days.
	,, 0–4.	Moderate growth 0·5–3, 9 days.
3. Ditto, but inoculated with small number of organisms .	Blue 0–26.	No growth, 1 day.
	,, 0–33.	,, ,, 4 days.
	,, 0–38.	,, ,, 9 ,,

diminishes progressively to a level situated between 30 and 40 mm. from the surface, below which the reducing activity of the medium is sufficient to render the conditions anaërobic. In the tube receiving the large inoculum the oxygen is used up by the organisms, even before macroscopic growth is evident, so that after 9 days no free oxygen is present in the medium below the band of growth. The tube receiving the small inoculum behaves like the control uninoculated tube ; no growth occurs, and the oxygen is not taken up by the organisms.

It may be concluded that oxygen diffuses relatively slowly into an agar medium, and that at about 30–40 mm. from the surface an equilibrium is reached, the oxygen being reduced by the medium as fast as it diffuses into it. In the presence of growing organisms the oxygen is used up very much more rapidly, even before macroscopic growth is evident, and the gaseous conditions below the level of growth are more or less anaërobic.

The Presence of Carbon Dioxide in Agar Shake Tubes.

It is conceivable that CO_2 in an agar shake tube might come from the air over the medium, or from the agar, or from the organisms themselves. Each of these possibilities was examined.

CO_2 Content of the Air over the Medium.

The normal CO_2 content of the outside air in a town is about 0·04 per cent. In the laboratory, however, where gas is being consumed by Bunsen burners,

incubators and water-baths, and where the windows frequently have to be kept shut, the air may contain as much as 0·1 per cent. This amount of CO_2 is too low of itself to permit growth of *abortus*. It seemed possible that in the incubator this amount might be exceeded, as the result of the evolution of CO_2 by growing cultures of different organisms. To test this, a series of eight estimations of the incubator air, taken as a rule first thing in the morning before the door had been opened, were made. The results varied from 0·09 to 0·17 per cent. CO_2, with a mean of 0·13 per cent. Even this figure is too low for the growth of *abortus*, and it must therefore be concluded that the CO_2 present at the site of growth in an agar shake culture cannot come wholly from the air over the medium.

Evolution of CO_2 from the Medium.

The method used for testing this possibility was as follows: 40 c.c. of 2·5 per cent. heart extract nutrient agar, pH 7·2–7·6, were delivered into each of 7 boiling tubes; the tubes were autoclaved, and then slanted in the usual way. When the agar had set, the tubes were placed in a closed metal jar, and a sample of the air was withdrawn for analysis. Seven control boiling tubes were filled each with 40 c.c. of tap-water; they were autoclaved and sloped in the same way as the agar tubes, so as to ensure that both sets should be exposed to similar atmospheric conditions; they were placed in another jar, the air of which was likewise analysed. Both jars were incubated at 37° C., usually for one to two days, and a second analysis made. The difference in the CO_2 percentage content of the air in the two jars afforded a measure of the CO_2 produced by the agar. The free air space in the experimental jar being known it was easy to calculate the actual volume of CO_2 given off by the agar.

Five experiments were carried out. Both with the agar and the control tubes there was a slight increase in the CO_2 content of the jar after incubation, due presumably to liberation of some of the gas taken up during the process of cooling. No difference was found between the two sets of tubes. It may be concluded, therefore, that no CO_2 is produced by agar during the first two days of incubation.

In other experiments it was found that if incubation was continued for two to three weeks, there was a definite excess of CO_2 in the jars containing the agar tubes. Since, however, growth in shake cultures usually occurs during the first few days, it cannot be dependent on this late production of CO_2 by the medium.

It was thought advisable to test the possibility of CO_2 production from the medium which Bang and Stribolt used in their original experiments. This is prepared by adding 0·75 per cent. agar and 5 per cent. gelatin to nutrient broth, and mixing the sterilized medium with half its quantity of sterile serum. In the experiments described in this paper horse-serum was used which had been preserved with chloroform. Before use it was freed from chloroform by evacuation. The results obtained were very striking. In two experiments it was found that during the first 24 hours of incubation an average of 6·75 c.c. CO_2 were evolved by seven tubes containing 40 c.c. agar, and during the first 48 hours 8·8 c.c. CO_2. From these figures it may be calculated that in a shake tube containing 8 c.c. of medium the CO_2 during the first two days should

at some time reach 2–3 per cent., depending on the rate of evolution and the rate of diffusion.

The evolution of CO_2 is in fact so abundant that it is possible to demonstrate a definite increase in the H-ion concentration of the medium by the usual colorimetric methods. In the depth of the medium the acidity increases, while in the upper 5 mm., owing to the loss of CO_2 from the surface, the reaction becomes more alkaline. Thus, in one experiment, the medium at the time of preparation had a reaction of pH 7·2. After incubation for one day the reaction had fallen to pH 7·0, except in the upper 5 mm., where it had risen to pH 7·8.

The mechanism responsible for the liberation of CO_2 from Bang's medium has not been exactly determined; it seems to depend, to some extent at least, on the presence in the serum of carbonate or bicarbonate, which is decomposed by the acid gelatin particles in the medium. The evolution of CO_2 is apparently very rapid, for a distinct change in the reaction of the medium can often be detected within an hour or less of its preparation.

It is thus seen that Bang's medium differs from nutrient agar in that a considerable amount of CO_2 is produced—sufficient in fact to raise the CO_2 content to 2–3 per cent. within 24 hours. This percentage is high enough to permit *Br. abortus* to multiply.

CO_2 Production by the Inoculated Organisms.

Preliminary experiments showed that during growth *Br. abortus* gave off a considerable quantity of CO_2. When grown on an agar slope in a $\frac{5}{8}$-in. tube, about 2 c.c. CO_2 were given off in three days—sufficient to raise the concentration of CO_2 in the air over the medium to about 15 per cent. It seemed not improbable, therefore, that a certain amount of CO_2 might be given off even by organisms which were not actively multiplying. This possibility was examined by two methods: (1) Agar slope cultures of *Br. abortus*, which had been incubated for two days in 5 per cent. CO_2, were washed off with Ringer's solution, and standardized by opacity to 2000 million organisms per c.c.; 4 c.c. of this suspension were introduced into each of seven large boiling tubes, which were then placed in a metal jar. A specimen of air was withdrawn from the jar and analysed for CO_2. Seven control boiling tubes, each containing 4 c.c. of sterile Ringer, were placed in a similar jar, the air of which was likewise analysed. Both jars were incubated for one to three days, after which the CO_2 content was again estimated. The difference in the CO_2 content of the two jars afforded a measure of the CO_2 produced by the organisms. Six experiments were performed in this way. The results showed that 1000 million organisms gave rise in an average of two days to 0·043 c.c. of CO_2. If these organisms were inoculated into a shake tube containing 8 c.c. of agar, the concentration of CO_2 would reach a figure of 0·54 per cent. Five similar experiments carried out under anaërobic conditions showed that 1000 million organisms produced 0·008 c.c. of CO_2, which in 8 c.c. of agar would give rise to a percentage of 0·1—a figure only about one-fifth of that reached under aërobic conditions. (2) Ringer suspensions of *Br. abortus* were prepared as before, and 2 c.c. were delivered into each of a number of Thunberg tubes of about 30 c.c. capacity. After incubation for one to three days the tubes were

attached to the Haldane apparatus, and a specimen of air withdrawn and analysed for CO_2. A control tube, containing no suspension, was always examined with each series of experimental tubes. Twelve examinations were carried out in this way, and it was found that the average amount of CO_2 produced in two days by 1000 million organisms was 0·033 c.c. This quantity in 8 c.c. of agar would give rise to a concentration of 0·43 per cent.

The results obtained by the two methods are sufficiently concordant, and indicate that even organisms which are not actively growing may yet give off a certain amount of CO_2. This is no place to discuss the mechanism governing the production of CO_2 by organisms which are apparently not multiplying; it is sufficient to refer to the numerous papers published in the *Biochemical Journal* by Stephenson, Quastel and Wooldridge during the past few years on the metabolism of so-called "resting bacteria" to learn that absence of growth, or even of viable cells, is not necessarily synonymous with absence of respiratory activity.

For the inoculation of agar shake tubes used in this work, about 1000 million organisms were introduced into 8–10 c.c. of agar. It may be calculated that these should give rise in the medium to a concentration of about 0·5 per cent. CO_2. Experiments indicated that the greater part of the CO_2 was given off by the organisms during the first 24 hours of incubation, so that it is probably fair to conclude that within a day after inoculation the organisms would be surrounded by a concentration of CO_2 just sufficient to enable growth to occur.

Consideration of the Three Sources of Carbon Dioxide.

Evidence has been brought to show that the air in the incubator may contain four times the amount of CO_2 present in the outside air; that little or no CO_2 is produced by sterile agar during the first two days of incubation; but that a considerable quantity of CO_2 may be given off by *abortus* bacteria, even when no apparent multiplication is occurring. In order for the organisms to start growing, it has been found that a concentration of about 0·5 per cent. CO_2 is required. If macroscopic growth is evident two days after inoculation, it is probable that multiplication starts within 24 hours, so that this concentration of CO_2 must be reached during the first day of incubation. Though a considerable quantity of CO_2 may be given off by sterile agar in two to three weeks, the quantity produced in 18 hours is too small of itself to allow of the initiation of growth. On the other hand, the CO_2 given off by the non-multiplying organisms is evolved mainly during the first day of incubation, and may reach a concentration sufficiently great to enable growth to commence.

It is not justifiable, however, to conclude that all the CO_2 given off by the organisms will remain in the medium. The actual percentage in the medium will be determined not only by the amount which is produced, but also by the concentration in the air of the tube. Diffusion into the agar from the air of the tube, and out of the agar into the air of the tube, will proceed until an equilibrium is reached. The higher the partial pressure of CO_2 in the incubator air, the greater will be the percentage of CO_2 in the medium.

That this reasoning is probably correct is indicated by the fact that if shake tubes seeded with *abortus* are incubated in a jar containing air which has been

freed from CO_2, the resulting growth is less than that in tubes incubated under the usual aërobic conditions. The process of diffusion of CO_2 through the agar, though apparently more rapid than that of oxygen, is none the less comparatively slow, so that the gas given off by the organisms during the first few hours of incubation will accumulate in sufficient quantity to permit growth to commence.

If the organisms are inoculated on to the surface of an agar slope, the CO_2 given off is bound to escape almost immediately, and even if the tube is sealed, provided the seal is not of such a character as itself to give off CO_2, the gas evolved by the organisms will not reach a sufficiently high percentage in the enclosed air to enable growth to occur. It is because the diffusion of the CO_2 away from the neighbourhood of the organisms is retarded by the agar, that growth is able to occur under aërobic conditions in a shake culture.

EXPLANATION OF THE ZONE PHENOMENON.

Having brought evidence to indicate that both oxygen and carbon dioxide are present in agar shake cultures of *Br. abortus* before growth has commenced, it remains to consider why the organisms develop only in one particular zone. Since optimal growth on surface cultures is obtained in an atmosphere of 20 per cent. oxygen and 5–10 per cent. CO_2, it is reasonable to conclude that in shake cultures growth will commence first in that zone of the medium in which the gaseous conditions most nearly approach the optimum. If the main supply of CO_2 is furnished by the organisms themselves, and if more CO_2 is given off in the presence than in the absence of oxygen, if follows that the concentration of CO_2 will be greater in the upper part of the medium, to which a certain amount of oxygen can penetrate, than in the lower part, where the conditions are anaërobic. At the surface of the medium, however, and immediately below it, the CO_2 given off will mostly escape into the air of the tube, so that the maximum concentration of this gas is likely to be reached a short distance below the surface. The oxygen, on the other hand, will be at its maximum concentration at the surface, and will diminish progressively with increasing distance from the surface. It seems probable, therefore, that the most favourable combination of gases will be present a short distance below the surface, at a point where the oxygen pressure is still relatively high, and where the CO_2 does not diffuse away too rapidly into the air of the tube. It is probably for this reason that the band of growth occurs about 2–4 mm. below the surface. Once the organisms have started to multiply in this situation, they consume all or practically all the oxygen diffusing into the medium, so that the conditions below the band are more or less anaërobic, and no further growth is possible beneath this level.

ADDITIONAL EVIDENCE BEARING ON THE ZONE PHENOMENON.

If these conclusions are correct, it should be possible to make certain deductions from them, and put these to the test of experiment. This has been done, and the following observations, it is believed, afford additional evidence of the essential truth of the conception which has been advanced.

Effect of the Size of the Inoculum on the Site of Growth.

If the CO_2 necessary for growth in nutrient agar is furnished chiefly by the organisms themselves, it follows that development will occur only when relatively large inocula are used. Small numbers of organisms will produce too little CO_2 to enable growth to commence, and the cultures will therefore remain apparently sterile. But if tubes inoculated with even very small numbers are incubated in an atmosphere containing added CO_2, then growth should occur satisfactorily. That this deduction is true is illustrated by the following experiment: A suspension of *Br. abortus* was prepared containing 1000 million organisms per c.c.; from this 1/350 and 1/123,000 dilutions were made in broth, and 0·1 c.c. of each dilution and of the original suspension was used for inoculating two sets of shake agar tubes, the medium being standardized to pH 6·1. One set was incubated aërobically, the other in air containing 10 per cent CO_2. The results are shown in Table II. It will be noticed that

TABLE II.—*Effect of Size of Inoculum on Position of Growth in Agar Shake Tubes. Results Recorded after Seven Days. Figures indicate distance of growth below surface in millimetres.*

Incubation.	Inoculum 0·1 c.c. of—		
	Original suspension.	1/350 dilution.	1/123,000 dilution.
Aërobically	Faint band, 4–6	No growth	No growth.
Air+10% CO_2	Thick growth 0–3	Semi-confluent growth 0–4	Multiple discrete colonies 0–24, larger above, smaller below.

in the aërobic series no growth occurred in the tubes seeded from the 1/350 and 1/123,000 dilutions. On the other hand, in the 10 per cent. CO_2 series a semi-confluent growth occurred in the tube seeded from the 1/350 dilution, while in the tube seeded from the 1/123,000 dilution multiple discrete colonies developed. The size of these colonies diminished steadily from above downwards; thus on the surface they were 1–2 mm. in diameter, in the 0–9 mm. zone they were visible macroscopically, while in the 9–24 mm. zone they could be seen only with a magnifying glass. This progressive diminution in size corresponded no doubt to a fall in the partial pressures of oxygen and CO_2 as the distance from the surface increased.

Another deduction may, however, be made. With a fairly large inoculum all the oxygen diffusing in from the surface is used up by the developing organisms, so that no growth is possible further down in the medium; if, however, a smaller inoculum is used, the oxygen should penetrate for some distance into the medium, and thus growth should occur, not in a narrow band 2–4 mm. below the surface, but throughout the upper 15 mm. or so, depending on the size of the inoculum. This likewise proves to be true, but for its demonstration it is necessary to use sufficiently large inocula to enable growth to occur under aërobic conditions. Thus when 0·1 c.c. of a suspension containing about 10,000 million organisms per c.c. was used for inoculating the

tubes, growth occurred in a band stretching from 2–6 mm. below the surface; when the same quantity of a suspension containing only 500 million organisms per c.c. was used, growth occurred in a band stretching from 3–12 mm. below the surface.

For bringing out this difference ordinary nutrient agar is not very suitable, since it presents too strong a barrier to the diffusion of oxygen. More satisfactory is the less solid medium originally used by Bang, the preparation of which has already been described. Preliminary experiments with methylene-blue showed that oxygen penetrated to a greater depth into this medium than into ordinary agar.

Since a considerable amount of CO_2 is evolved by Bang's medium, it is unnecessary to incubate the tubes in an atmosphere containing added CO_2, as is essential with agar if growth is to result from small inocula.

A suspension of *Br. abortus* was prepared containing 1000 million organisms per c.c.; from this 1/350 and 1/123,000 dilutions were made in broth, and 0·1 c.c. of each dilution and of the original suspension was used for inoculating $\frac{3}{4}$ in. tubes containing about 15 c.c. of Bang's medium standardized to pH 7·0. The tubes were incubated aërobically. A duplicate set of tubes containing about 1/200,000 methylene-blue were similarly inoculated. The results are given in Table III, and are self-explanatory. In the methylene-

TABLE III.—*Effect of Size of Inoculum on Position of Growth. Results Recorded after Four Days. Figures indicate distance below surface in millimetres.*

	Inoculum 0·1 c.c. of—		
	Original suspension.	1/350 dilution.	1/123,000 dilution.
Shake tubes of Bang's medium	Band of growth 6–8	Faint band of growth 10–13	Multiple tiny discrete colonies 10–32, larger above, smaller below.
Ditto, coloured with methylene blue	Blue 0–5 Faint band of growth 5–9	Blue 0–10 Faint band of growth 10–13	Blue 0–23. Multiple tiny discrete colonies 12–25, larger above, smaller below.

blue series of tubes growth was slightly less abundant; this has been a constant finding, and is presumably due to an inhibitory effect of the dye on growth. There is an almost perfect correspondence between the depth to which the methylene-blue remains coloured and the position of the growth, indicating that no growth is possible in the absence of oxygen. In the tubes inoculated with the highest dilution, the greater size of the discrete colonies nearer the surface, where oxygen was more abundant, and their smaller size near the lower margin of growth, where oxygen was scanty, also indicates the importance of oxygen in favouring growth.

It may be wondered why, if CO_2 is given off by Bang's medium, growth does not occur at the surface. The explanation of this seems to be that the upper 5 mm. or so of the medium are markedly alkaline (see p. 156) and little or no free CO_2 exists in this zone.

The great superiority of Bang's serum agar gelatin medium over nutrient

agar is evident from a comparison of Tables II and III, and is no doubt attributable to the CO_2 given off by the serum. It is interesting to note that Stribolt found serum to be essential for the growth of *Br. abortus*; in agar gelatin medium without serum no growth occurred. Since he was clearly using inocula containing small numbers of bacilli, this result is perfectly intelligible.

Effect of the Reaction of the Medium on the Site of Growth.

Since carbon dioxide remains free in an acid medium, but enters into combination to form carbonates in an alkaline medium, it follows that the CO_2 given off by the inoculated organisms in a shake culture will attain a higher concentration in an acid than in an alkaline medium. Growth should therefore occur more readily when the reaction is acid than when it is alkaline. Numerous experiments have been performed in which three batches of medium, standardized to pH 6·0, 7·0 and 8·0, have been compared. The results were quite uniform, showing that growth always occurred best in the acid medium, and failed completely in the alkaline medium; in the neutral medium growth was rather poorer than in the acid medium.

But a further deduction may be drawn. Since, owing to the favouring effect of oxygen, the organisms tend to develop as near the surface as is consistent with the maintenance of a sufficiently high concentration of CO_2, growth should occur nearer the surface in an acid than in a neutral or slightly alkaline medium. The reason for this is that the concentration of CO_2 required to enable growth to occur must be maintained up to a point nearer the surface when the medium is acid than when it is alkaline. Comparison of media standardized to pH 6·0 and pH 7·0 has shown the truth of this deduction. Three experiments may be cited. Using comparable inocula, in one experiment growth in the acid medium occurred 3–6 mm., and in the neutral medium 4–10 mm. below the surface. In another experiment the zone in the acid medium was 3–5 mm., in the neutral medium 6–8 mm. below the surface. And in a third experiment the figures were 2–7 mm. for the acid, and 8–12 mm. for the neutral medium.

The conclusion to be drawn is that growth occurs more abundantly and nearer the surface in an acid than in an alkaline medium.

Effect on the Site of Growth of Incubation in Different Atmospheres.

If the band of growth in shake cultures incubated aërobically is determined by the factors already described, it should be possible to alter its position by replacing the air over the tube with different combinations of gases. Thus, if the cultures are incubated in air containing 5–10 per cent. CO_2, growth should occur on and immediately below the surface, and should be very much more abundant. If they are incubated in pure oxygen, growth should occur slightly lower in the medium than under aërobic conditions, and should commence at a level at which the optimum concentration of 20 per cent. is attained; moreover, on account of this higher concentration of oxygen, growth should likewise be more abundant. Under a lowered pressure of oxygen, growth should be less abundant than under aërobic conditions, and should occur only

in a narrow zone situated close to the surface. Under anaërobic conditions there should be no growth at all.

A number of experiments were performed using comparable inocula ; the general type of result which was obtained may be summarized as follows :

Incubation.	Growth.
Aërobically . . .	Faint band, 2–6 mm. below surface, after 3 days.
Air + 5 per cent. CO_2 .	Thick band, 0–3 ,, ,, ,, ,, 3 ,,
99 per cent. O_2 . .	Fairly thick band, 3–8 mm. below surface, after 3 days.
4 per cent. O_2 . .	Very faint band, 2–3 mm. below surface, after 8 days.
Anaërobically . .	No growth.

It will be noted that the actual results are similar to those predicted.

It might be expected that in a shake culture incubated in pure oxygen growth should occur in a zone stretching for a considerable distance below the surface. This is true if a small inoculum is used, but if the tubes are seeded with 0·1 c.c. of a suspension containing about 5000–10,000 million organisms per c.c.—the inoculum which has most commonly been used in this work—growth occurs in the form of a wide band, whose upper margin is 2–3 mm., and whose lower margin is generally 8–12 mm. below the surface. Beneath this level no growth is visible, because all the oxygen diffusing into the medium is used up by the organisms. The curious spectacle is presented of a tube, incubated in pure oxygen, in which within about 1 cm. of the surface the conditions are strictly anaërobic. The following experiment demonstrates this clearly: Two shake agar tubes were coloured with methylene-blue and one of them was inoculated with 0·1 c.c. of a suspension of *abortus* containing about 5000 million organisms per c.c. The tubes were incubated in a closed jar filled with oxygen. The results were as follows :

Control uninoculated tube .	Blue 0–45 mm. below surface after 1 day.
	,, 0–55 ,, ,, ,, ,, 2 days.
	,, 0–60 ,, (bottom) below surface after 3 days.
Inoculated tube . . .	Blue 0–16 mm., with poor growth 2–8 mm., after 1 day.
	Blue 0–3 mm., with poor to moderate growth 2–10 mm., after 2 days.
	Blue 0–3 mm., with moderate growth 2–10 mm. (maximum 4–6 mm.) after 3 days.

In the control tube the oxygen had penetrated to the bottom within 3 days, whereas in the inoculated tube the free oxygen was limited to a narrow zone at the extreme summit of the agar column.

It may be concluded that both the amount and the position of the growth in shake cultures are influenced by incubation under different gaseous conditions in a way which accords with that theoretically anticipated.

THE DOUBLE ZONE PHENOMENON.

Though not falling within the deductions to be tested experimentally, it seems advisable to draw attention to a phenomenon which has not infrequently been encountered, and whose explanation is rather puzzling.

Stribolt's results, as described in Bang's original paper, may be briefly recalled. Using the serum agar gelatin medium, and incubating the shake tubes in about 90 per cent. oxygen, he noticed that growth sometimes occurred in two distinct zones—an upper one situated 3–13 mm., and a lower situated about 30–40 mm. below the surface ; between the two zones there were merely a few discrete colonies. The phenomenon was not constant in its appearance ; it seemed to depend to some extent on the amount of serum in the medium, and the number of air bubbles which were included when the agar gelatin medium was mixed with the serum.

So far as I am aware, no one has ever confirmed these results ; my own attempts to repeat them exactly have likewise proved unsuccessful. It may be noted that Stribolt seeded his tubes with uterine exudate, not with suspensions of pure cultures, and as growth did not occur for several days, it is probable that the number of inoculated organisms was small.

Though the exact type of picture recorded by Stribolt has not been reproduced, it has been noticed on several occasions that a secondary band of growth may develop some distance below the primary band. The secondary band develops more slowly, is generally narrow, and is always very much fainter than the primary band. The distance separating the two bands depends on the type of medium used and the size of the inoculum. This double zone phenomenon has been noticed in tubes incubated under the most varied atmospheric conditions, as is seen in Table IV. In order to avoid overcrowding

TABLE IV.—*Conditions under which the Double Zone Phenomenon was seen. Figures indicate distance below surface in millimetres.*

Incubation.	Medium.	Primary band.	Secondary band.
Aërobic	Agar	Slight growth 0·5–2	Faint ring at 6 mm.
,,	Bang	,, ,, 0·5–2	,, ,, 12 ,,
Air + 0·5% CO_2	Agar	Moderate growth 0–2	,, ,, 8 ,,
Air + 5% CO_2	,,	Thick growth 0–2	,, ,, 5 ,,
99% O_2	,,	,, ,, 1–3	,, ,, 15 ,,
99% O_2	Bang	Moderate growth 3–6	,, ,, 11 ,,
99% O_2	,,	,, ,, 6–9	,, ,, 17 ,,
99% O_2	,,	Slight growth 1–3	Faint band 16–22 mm.
99% O_2 + 1% CO_2	,,	,, ,, 3–5	,, ,, 12–15 ,,
95% O_2 + 5% CO_2	Agar	Thick growth 1–3	,, ring at 15 mm.

of the table, a number of relevant data have been omitted ; a study of these, however, suggests that the double band phenomenon is most common when a moderate-sized inoculum is used, insuring that the primary growth is not too abundant, and when the pressure of oxygen is not less than that present in the atmosphere ; a certain amount of added CO_2 likewise seems to be beneficial.

In seeking the explanation of this phenomenon, it was natural to suspect that some inhibitory substance was formed by the organisms in the primary band, which prevented growth for some distance lower in the medium. Experiments with indicators, such as phenol red and bromo-thymol blue incorporated in the agar, showed that at the site of growth, and for 5–10 mm. or so below it, there was a considerable increase, amounting to 0·4 or 0·6 pH, in the alkalinity of the medium. Evidence has already been brought to show that growth in agar shake cultures of definitely alkaline reaction is very slight or altogether absent, because the CO_2 liberated by the organisms combines to form alkaline carbonates. If, then, alkali is produced by the organisms during growth, and diffuses for some distance above and below the band, it is reasonable to suppose that it will serve to diminish the concentration of CO_2 in the locality. This observation may furnish the clue to the explanation of the double-band phenomenon. Admitting that the phenomenon is most common with a moderate-sized inoculum, and in the presence of a high partial pressure of oxygen, it may be supposed that the organisms situated in the most favourable zone some distance below the surface commence to multiply first. As the result of their growth, alkali is produced, which combines with any free CO_2 in the immediate neighbourhood of the growth, and for a distance of 5–10 mm. below it. Since the primary growth is not very heavy, not all the oxygen diffusing into the medium is consumed by the organisms ; some passes down for a variable distance into the medium. The partial pressure of oxygen beneath the band is, of course, very low, but it might suffice to allow the organisms lower down in the medium to multiply, if only there was an adequate concentration of CO_2 available. No free CO_2, however, is present till the lower margin of the alkaline zone is reached. At this level both CO_2 and oxygen may be available, and therefore the organisms may multiply, giving rise to a faint secondary band of growth.

Whether this is the correct explanation or not it is impossible to say. If it is, and if it is granted that the double zone phenomenon originally observed by Stribolt is essentially the same as that just described, it follows that the explanation of Stribolt's results given by Bang, namely that the phenomenon indicates the existence of two optimum partial pressures of oxygen for the growth of *Br. abortus*, must be wrong. Numerous experiments with surface cultures have been made to test the truth of Bang's conclusions, but no evidence has been obtained to suggest that there is a high and a low optimum partial pressure of oxygen ; all the results show, on the other hand, that the optimum oxygen pressure is in the neighbourhood of 20 per cent.

SUMMARY AND CONCLUSIONS.

1. In agar shake cultures incubated aërobically CO_2-sensitive strains of the bovine type of *Br. abortus* grow in the form of a band situated about 2–5 mm. below the surface.

2. The early part of the paper is concerned with providing an explanation for this zone phenomenon of growth.

3. In order for growth to occur, both oxygen and CO_2 are required. The oxygen diffuses into the medium from the air ; the CO_2, it is believed, is mostly

derived from the inoculated organisms themselves before actual growth commences.

4. Growth first begins in a zone situated as near the surface as is compatible with the existence of an adequate concentration of CO_2.

5. Growth does not occur at or immediately below the surface, because the CO_2 liberated from the organisms diffuses too rapidly into the air of the tube ; nor does it occur low down in the medium, because the oxygen diffusing into the agar is used up by the organisms near the surface, so that the conditions below the band of growth are practically or completely anaërobic.

6. In the latter part of the paper, additional evidence is advanced to support the essential truth of this conception. It is shown that the amount of growth and the position of the band are altered by variations in the size of the inoculum, in the reaction of the medium, and in the gaseous conditions under which the cultures are incubated, in a manner which is in harmony with the explanation offered.

7. The double zone phenomenon, originally described by Bang, is considered, and reasons are brought to doubt the truth of Bang's deductions of the existence of two optimum oxygen pressures for growth.

A different explanation is offered, depending on the production of alkali by the organisms in the primary band.

In conclusion I should like to express my thanks to the Government Grant Committee of the Royal Society for financial assistance in this investigation.

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COMPLEMENT-FIXATION WITH VACCINIA CULTURE-VIRUS.

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Received for publication May 8th, 1931.

THE reaction of complement-fixation has frequently been demonstrated with the serum of the rabbit, calf and man infected with vaccinia, and with human serum from cases of smallpox, using as antigen either vaccine-lymph, crusts or pustular material from smallpox or extracts of them. The literature has been reviewed by Gordon (1925), Gildemeister (1927) and Sobernheim (1925, 1927). Gordon (1925) described a flocculation reaction, using crusts

from smallpox and vaccinia-immune rabbit serum ; it has been further investigated as a diagnostic method by Burgess, Craigie and Tulloch (1929). Bedson and Bland (1929) obtained complement-fixation with hyperimmune guinea-pig serum and virus from guinea-pig or rabbit tissue. The "*kokto-antigen*" prepared by boiling infected brain used by Kraus and Takaki (1925), Takaki, Bonis and Koref (1926) appears to be too non-specific to be satisfactory (Michalka, 1927 ; Gildemeister and Heuer, 1928). The suitability of unheated infected brain as an antigen is still in doubt, although Wollman and Urbain (1928), who used it, have reported significant fixation with immune guinea-pig serum. "*Koktoantigen*" prepared from vaccine lymph appears to be more satisfactory (Lurje and Wolkowitsch, 1928 ; Glaser and Koref, 1928).

Although none of these antigens has been free from bacteria (with the possible exception of brain), it has nevertheless been held that specific union of virus and antibody has been demonstrated by these methods, and that the reactions have indicated the antigenic similarity of the viruses of variola, alastrim and vaccinia—a view that seems to be amply justified. Schultz (1928) and Schultz, Bullock and Lawrence (1928), on the other hand, from a review of the literature and the results of their own work, have regarded all such findings as due to concomitant bacterial antigens and corresponding antibodies. They considered that controls adequate to eliminate this source of error had not been made. Burgess, Craigie and Tulloch (1929), in meeting this criticism, have shown that the staphylococci contaminating the crusts of vaccinial lesions did not account for flocculation. Bedson and Bland (1929) did not obtain complement-fixation with anti-vaccinial serum, using as antigen a suspension of organisms cultured from their virus material aërobically on agar.

I. COMPLEMENT-FIXATION WITH IMMUNE RABBIT SERUM.

The cultivation of vaccinia virus (Maitland and Maitland, 1928 ; Maitland and Laing, 1930) afforded an opportunity to secure as antigen, culture-virus free from bacteria or their products, and furnished a direct approach to the question, Does complement-fixation result from the interaction of virus and antibody ? The experiments in this section are concerned with demonstrating by the use of this culture-virus the specific nature of complement-fixation resulting from the interaction of vaccinia virus and immune rabbit serum. The value as antigens of government-lymph and culture-virus have been compared, and several points relating to the complement-fixing property of rabbit serum have been studied.

Technique.

A dropping method has been used throughout, the total volume in the test being 20 drops (approximately 1 c.c.).

Antigens.—(*a*) Government-lymph : A 2 per cent. suspension in saline from which the larger particles had been allowed to settle.

(*b*) Culture virus : The medium consisted of minced rabbit kidney 3 per cent., rabbit serum 30 per cent. and Tyrode's solution 67 per cent. The

cultures after incubation were ground with sand, the whole then thoroughly mixed, the larger particles allowed to settle and a 2 per cent. suspension of the upper layer made in saline.

Antisera were used in dilutions of 1 in 10, 1 in 25 and 1 in 50, and the serum control in a dilution of 1 in 10. All were inactivated by exposure to 55° C. for 30 minutes.

Hæmolytic system.—A 2·5 per cent. suspension of sheep's red blood-cells has been used throughout. In titrating hæmolysin pooled guinea-pig serum was used in a dilution of 1 in 10, and the result read after incubation in the water-bath at 37° C. for 1 hour. 3 M.H.D. hæmolysin have been used in all fixation and complement-titration tests.

Complement.—This has been titrated against 3 M.H.D. of hæmolysin, and the reading made after incubation for one hour in the water-bath at 37° C. The amount used in the tests has usually been 4 M.H.D.

The test.—5 drops each of serum, complement and antigen were mixed and allowed to fix for 15 to 20 hours at about 4° C. Then after adding 5 drops of sensitized cells (equal volumes of 5 per cent. cells and 6 M.H.D. hæmolysin) the tubes were incubated at 37° C. in a water-bath for one hour. Final readings were made after standing overnight in the ice-chest.

Controls.—In addition to the usual serum and antigen controls, normal rabbit serum has always been included. In the tests with normal serum hæmolysis was usually completed in 15 to 20 minutes. Positive results with immune sera were not accepted if the hæmolysis in their serum-controls lagged behind that of the serum-control of the normal serum. Occasionally a sample of rabbit serum was so anti-complementary that it was useless as a control.

Discussion of Technique.

The technique described was adopted after preliminary trials with a number of modifications. The observation of Bedson and Bland (1929) that rabbit serum is markedly anti-complementary has been confirmed; it is much more anti-complementary than human serum, but the technique described has given satisfactory results. All attempts to obtain fixation with these materials by the method employed by Gordon (1925), *i. e.* 30 minutes at 37° C., have been unsuccessful.

Attempts were made to obtain positive results with fixation for one hour at 37° C. by increasing the sensitivity of the test as follows : (*a*) Cholesterinizing the antigen, 1 per cent. of a 1 in 100 alcoholic solution of cholesterol being added to 2 per cent. Government-lymph. It was found, however, that this antigen gave fixation with normal rabbit serum. This difficulty might not apply to reactions made with human serum, or the serum of some other animals. Mackie and Finkelstein (1928) have noted that the serum of the rabbit, ox, horse and sheep generally react with cholesterol to fix complement, even though they have been inactivated, while inactivation of the serum from man, the guinea-pig and white rat removes this property.

(*b*) Formalizing the antigen : The introduction of formalin into the antigen-antibody system, which has been reported by Reyner (1928) to make a complement-fixation test more delicate, was found in this work to increase the

anti-complementary properties of the test materials to such an extent that readings were impossible, even when the proportion of formalin in the test was reduced to 1 part in 28,000.

Experimental.

The sera of 16 rabbits which had been infected intradermally with culture-virus have been tested for complement-fixation, using culture-virus as antigen. Fixation has been obtained in every instance, and the results are shown in Table I. These sera were obtained from rabbits that had been used for

TABLE I.—*Complement-Fixation with the Sera of Rabbits Infected with Culture-Virus, using Culture-Virus or Government-Lymph as Antigen.*

No. of rabbit.	Interval between infection and collection of serum.	Virus used as antigen.	Serum dilutions. 1 in 10.	1 in 25.	1 in 50.
L 94	9 months	C. V.	++	+	0
L 97	8½ ,,	,,	+	0	0
L 98	8½ ,,	,,	+	0	0
L 136	36 days	,,	+++	++	+
		G. L.	++++	++++	+++
L 139	30 ,,	C. V.	+	0	0
		G. L.	++++	+++	+++
L 141	10 ,,	C. V.	+	0	0
		G. L.	++++	+++	+++
L 150	11 ,,	C. V.	++	++	+
L 180	10 ,,	C. V.	++	+	0
		G. L.	+++	+	0
L 185	12 ,,	C. V.	+	+	0
		G. L.	+++	++	0
L 186	11 ,,	C. V.	+++	++	+
		G. L.	++++	++++	+++
L 187	14 ,,	C. V.	++	++	0
		G. L.	++++	++++	+++
L 190	10 ,,	C. V.	+	0	0
L 196	13 ,,	,,	+	0	0
L 197	13 ,,	,,	++	+	+
L 198	15 ,,	,,	+	0	0
L 199	16 ,,	,,	+	0	0

++++ = Complete fixation. +++ = Almost complete fixation. ++ and + = Degrees of partial fixation. 0 = No fixation. C. V. = Culture-virus. G. L. = Government-lymph.

titrating culture-virus, and were selected because they had three or more well-developed lesions. The results demonstrate clearly the specific fixation of complement in the reaction between virus and corresponding antibodies. The system was free from bacteria ; the culture material from which the antigen was derived contained rabbit serum and rabbit tissue ; the antiserum was obtained from the same species.

It will be noticed that complement-fixing antibodies were detectable as early as 10 days after infection, and Table II shows that they could be detected for at least as long as 8½ to 9 months. There was a marked variation in the

TABLE II.—*Persistence of Complement-Fixing Antibodies in the Serum of Rabbits Infected with Culture-Virus, using Government-Lymph as Antigen.*

No. of rabbit.	Interval between infection and collection of serum.	Serum dilutions. 1 in 10.	1 in 25.	1 in 50.
L 94	51 days	++++	+++	++
	23 weeks	+++	+++	++
	9 months	+++	+	0
L 97	42 days	+++	+++	+
	22 weeks	++	+	0
	8½ months	++	0	0
L 98	40 days	+++	+	0
	22 weeks	++	0	0
	8½ months	+	0	0

Symbols as for Table I.

response of individual rabbits. The strength of the antibody at any period appeared to depend upon the ability of the rabbit to respond and not entirely upon the severity of the infection, *i. e.* the number of lesions or the development of generalized vaccinia. The persistence of antibodies may be related to an observation of Olitsky and Long (1929), who recovered vaccinia virus from the tissues of rabbits for as long as 133 days after dermal infection.

A comparison of the value of Government-lymph and culture-virus as antigen was made by testing seven sera with both antigens at the same time (Table I). In every instance the amount of fixation was greater with Government-lymph, but the advantage was most evident with those sera that gave a weak reaction with culture-virus. The reason for this is not apparent; the superiority of Government-lymph did not correspond to a proportionally greater content of living virus. It may have been related to differences in the number or kind of particles in the antigen or to the attachment of the virus to them. The infective titre of the culture-virus was about 1/15,000, and that of Government-lymph about 1/20,000 (0·2 c.c. inoculated intradermally on the back of rabbits).

It is of interest that the vaccinia virus had maintained its antigenic properties in culture. The material used for the *in vitro* tests was obtained from the 17th to the 24th culture-passages (Maitland and Laing, 1930).

That the results obtained with culture-virus were in fact due to the presence of vaccinia virus in it was supported from experiments in which the serum of four rabbits that had been infected with Government-lymph gave specific fixation with culture-virus as antigen. The reactions were similar to those obtained with serum from rabbits infected with culture-virus (partial fixation in a dilution of 1 in 10, *cf.* Table I); there was one exception, a hyperimmunized rabbit whose serum produced almost complete fixation in a dilution of 1/25.

II. COMPLEMENT-FIXATION WITH SERUM FROM CASES OF SMALLPOX.

The reaction of complement-fixation with serum from cases of smallpox, using culture-virus as antigen, has been demonstrated in a small series of tests, and a comparison has been made of the antigenic value of culture-virus and Government-lymph for testing such sera.

(*a*) *Tests with Culture-virus as Antigen.*

The technique was similar to that already described, with the following modifications: 2 M.H.D. of complement and 2 M.H.D. of hæmolysin were used; the antigen had an infective titre of 1/390,000 and was used in a strength of 4%; three dilutions of serum were tested, 1 in 2, 1 in 5 and 1 in 10; fixation was carried out as before. Pooled Wassermann-negative sera was used as control. It will be seen from Table III that complement-fixation occurred with each of eight sera; one other, not shown, was anti-complementary. The control serum was negative.

(*b*) *Tests with Government-lymph as Antigen.*

Several modifications of the technique previously used were tried (variations in the hæmolytic system and in fixation), but the best results were obtained with the following: 2·5 M.H.D. complement; 3 M.H.D. hæmolysin; fixation at room-temperature for 17 hours (overnight). The antigen had an infective titre of about 1 in 20,000, and was diluted 1 in 50. Three Wassermann-negative sera, used as controls, were negative.

TABLE III.—*Complement-Fixation with Serum from Cases of Smallpox.*

No. of serum.	Antigen.	Dilution of serum.				
		1 in 2.	1 in 4.	1 in 5.	1 in 10.	1 in 20.
1	C. V.	++	..	++	+	..
2	,,	++++	..	++++	++++	..
2	G. L.	..	++++	..	++++	++++
3	C. V.	+	..	+	±	..
3	G. L.	..	++++	..	++	0
4	C. V.	+	..	+	+	..
5	,,	++	..	++	++	..
6	,,	++	..	++	++	..
7	,,	+++	..	++	++	..
8	,,	+++	..	+++	+++	..
9	G. L.	..	+	..	0	0
10	,,	..	0	..	0	0
11	,,	..	++++	..	+++	+
12	,,	..	+++	..	++	++

Symbols as for Table I.

Of four sera tested (Table III), three were positive and one (No. 10) was negative. The test with serum No. 3 again indicated the superiority of Government-lymph with a serum that reacted weakly with culture-virus.

All these sera were obtained from cases of " mild " smallpox or alastrim, and were collected from 2 to 27 days after the appearance of the rash. The cases varied in the extent of their lesions but there was no correlation between this, or the time when the serum was collected and the amount of complement-fixation. As was noted for rabbits, there was a marked individual difference in antibody response. The serum from Cases 9 10, 8 and 12 were collected on the 2nd, 3rd, 4th and 7th days respectively, the others from the 16th to the 27th day (Table III). Nevertheless of twelve sera tested, only one failed to fix complement.

These results agree with what has been established by others, viz. that complement-fixing antibodies are almost always present in the serum of cases of smallpox, and that their amount does not depend on the extent or severity of the infection. As a diagnostic test, complement-fixation appears to have as good a claim as any other serological reaction. Its performance requires a less involved procedure than the flocculation reaction, which necessitates the collection and preparation of smallpox crusts as well as immune rabbit serum, and the results appear to be as frequently positive. The use of culture-virus as antigen would ensure a reagent of fairly uniform composition, having the additional advantage of being free from bacteria.

SUMMARY AND CONCLUSIONS.

1. Vaccinia culture-virus after 17 to 24 passages in a medium consisting of minced rabbit kidney, rabbit serum and Tyrode's solution was a satisfactory antigen for complement-fixation tests.

2. Complement-fixation was obtained with serum of rabbits infected with culture-virus, or Government-lymph and with human serum from cases of alastrim, using culture-virus as antigen.

3. In the tests with culture-virus and rabbit serum, the reagents having been free from bacteria and the antigen and antiserum having been obtained from one species, the results demonstrate beyond question the specific union *in vitro* of vaccinia virus and a corresponding antibody.

4. The complement-fixing property of the serum of rabbits was demonstrable as early as 10 days after infection and persisted for at least nine months.

5. The amount of complement-fixing antibody did not depend upon the extent of infection, either in rabbits or in persons suffering from alastrim.

6. Vaccine-lymph (Government-lymph) was in some instances a better antigen than culture-virus ; this did not depend entirely upon its content of living virus, as judged by its infective titre.

7. Each of eight sera from cases of alastrim fixed complement with culture-virus as antigen, and three of four sera with Government-lymph as antigen.

8. The complement-fixation reaction appears to have as good a claim as any other serological reaction to be used as a diagnostic test for smallpox.

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STUDIES ON PROTEIN-FREE SUSPENSIONS OF VIRUSES. I.—THE ADSORPTION AND ELUTION OF BACTERIOPHAGE AND FOWL-POX VIRUS.

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Received for publication May 14th, 1931.

THE properties of filterable viruses are often obscured by the masking effect of associated proteins. The character of the electrical charge, the resistance to heat and chemicals as well as other characteristics may conceivably be modified by the proteins usually present in virus suspensions. It seemed desirable, therefore, for the purpose of studying the reactions and behaviour of pure viruses, to obtain them in suspensions free of proteins. The object of the present paper is to describe a method which has enabled us to obtain suspensions of bacteriophage and fowl-pox virus free from proteins.

The principle underlying the procedure is that of adsorption followed by specific elution. Willstaetter (1922) was the first to obtain protein-free enzymes by means of adsorption and elution after autolysis of the cell proteins. Fodor and co-workers (1925 ; 1926) went a step further and obtained enzymes free from proteins by means of adsorption followed by specific elution. Recently Frankel and Olitzki (1930) have successfully applied this principle towards obtaining antibodies free from proteins, and we have found that under appropriate conditions it is possible also to apply it to the so-called filterable or ultraviruses.

The adsorption of virus by a variety of adsorbants has been studied by a number of investigators [Kraus and his co-workers (1909), Levaditi and Nicolau (1923), Ledingham (1927), Lewis and Andervont (1927)], but the only data we have found bearing on the elution of virus from the adsorbate are contained in the papers by Gildemeister and Herzberg (1924) and by Callow (1927), both dealing with bacteriophage. The former adsorbed the phage with Kieselguhr by mixing 10 c.c. broth-phage diluted 1 : 100 with 0·2 gm. of the powder and shaking for 3 minutes ; to the adsorbate they added N/25 to N/100 ammonium solution, allowing the mixture to stand for 10 minutes. By this procedure they claim to have recovered a large part of the phage. Callow used as adsorbant kaolin which had been boiled in HCl and then washed free of acid, 2·5 c.c. of phage being adsorbed with 0·2 gm. kaolin. The adsorption was more complete at pH 8·4 than at pH 6·4. The sediment was then eluted with M/250–M/500 K_2HPO_4, Na_2SO_4 or Na_2CO_3, and the phage recovered in the eluting fluid. Neither of the authors states whether the eluted phage suspension obtained by them was free from protein.

In our experiments we used kaolin as the adsorbant and tested a variety of eluents. Specific elution does not apparently follow any given law, and a suitable eluent can only be found by trial. For example, glycocol solutions, which proved satisfactory for eluting enzymes and antibodies, gave negative results with either phage or fowl-pox virus. Ammonia, in suitable concentrations, is the only eluent thus far tested which has yielded positive results.

ADSORPTION AND ELUTION OF BACTERIOPHAGE.

After a number of experiments it was found that the following procedure gave dependable results. One c.c. of a broth phage was added to 1·0 c.c. saline containing 0·5 gm. kaolin. The kaolin suspension was sterilized in the autoclave and cooled before the phage was added. The phage-kaolin mixture was shaken thoroughly for ten minutes and placed in the ice-box overnight. The following morning the suspension was again shaken, and then centrifugalized at 2000–2500 revolutions per minute until the supernatant fluid was clear. The supernatant fluid thus obtained gave a negative reaction with Esbach and was practically free of phage. After decanting and draining the supernatant fluid, the sediment was resuspended in 4·0 c.c. N/100 $NH_4(OH)$, and again left in the ice-box overnight. The next day the suspension was centrifugalized, the supernatant fluid decanted, neutralized and tested for phage and protein. As a rule the fluid was free of even traces of protein, while the phage content was at least 50 per cent. of that of the original broth.

TABLE I.—*Adsorption of Phage by Kaolin after 24 Hours. (Colonies per cubic centimetre in millions.)*

	Per cent. kaolin.			
	5	10	20	33
(A) Kaolin suspended in saline and autoclaved :				
Kaolin sediment resuspended in initial volume	2·0	3·3	9·0	18·0
Supernatant fluid	20·0	23·0	1·7	0·0
(B) Kaolin sterilized dry :				
Kaolin sediment	3·4	1·6	15·0	20·0
Supernatant fluid	40·0	40·0	0·3	0·0

TABLE II.—*Elution with 2 per cent. Glycocol Solution in Varying Salt Solutions.*

No.	Per cent. glycocol.	Per cent. NaCl.	Phage colonies, millions per c.c.
1	2·0	0·0	0·8
2	2·0	0·25	0·18
3	2·0	0·75	0·9
4	2·0	1·0	0·07
5	2·0	2·0	0·04
Kaolin sediment	0·0	0·75	12·5
Broth control	0·0	0·75	13·5

TABLE III.—*Elution with HCl, NaOH and NH_4OH.*

	HCl.		NaOH		NH_4OH		Control.
	N/10.	N/100.	N/10.	N/100.	N/10.	N/100.	
	Millions of phage colonies per c.c.						
Kaolin sediment	0·0	0·0	0·0	3·2	20·0	12·0	..
Eluate	0·0	0·0	0·0	10·0	80·0	90·0	..
Broth	..	..	..	..	..	..	80·0

TABLE IV.—*Adsorption and Elution of Phage after the Addition of Serum to the Bouillon.*

(A) 1 part broth phage.
1 ,, saline.
0·5 ,, kaolin.

(B) 1 part broth phage + 0·05 c.c. normal serum.
1 part saline.
0·5 ,, kaolin.

Adsorbed 24 hours ; fluid free from protein.

Nature of eluate.	Phage concentration in— Eluate.	Kaolin sediment before elution.	Control.
	Millions of phage colonies per c.c.		
(A) Broth :			
1. Eluted with N/100 NH_4OH	24·0	50·0	..
2. Eluted with H_2O	1·9	..	..
(B) Broth plus serum :			
3. Eluted with N/100 NH_4OH	48·0	60·0	..
4. Eluted with H_2O	2·2	..	..
5. Broth phage + 0·05 c.c. serum	..	..	80·0

Tables I–IV present summaries of typical experiments indicating the results obtained by different procedures. It is apparent from these experiments that the rate and degree of adsorption depends on the concentration of kaolin, and the elution on the nature of the reagent used. Strong acid or alkaline solutions destroy the phage, but weak alkaline solutions act as eluents, removing a larger proportion of the phage from the kaolin, while at the same time leaving the protein behind.

The concentration of the bacteriophage at the various stages of the process of adsorption and elution was determined by the plate method of Prausnitz and Firle (1924), counting the number of "holes," as well as by dilution in broth. In all of our experiments a coli-phage was used.

ADSORPTION AND ELUTION OF FOWL-POX VIRUS.

The adsorption and elution of fowl-pox virus was effected in the same manner as with phage. Twenty per cent. kaolin adsorbed protein and virus completely in 24 hours. The virus was not inactivated by this concentration of kaolin, and could be partially recovered by elution with N/500 NH_4OH. Higher concentrations of ammonia destroyed the virus, indicating that it is more sensitive to the action of alkalis than is the coli phage used in our experiments.

The following are typical experiments illustrating the technique employed and the results obtained:

Experiment 1.—Dry virus material was ground up in saline (0·1 gm. dry virus in 5 c.c. saline), and 5 c.c. of the stock virus suspension added to 10 c.c. saline containing 5·0 gm. kaolin. The mixture was shaken thoroughly and placed in ice-box overnight. Next day the mixture was centrifugalized. The supernatant fluid gave a negative Esbach reaction.

The stock virus suspension, the kaolin sediment and the supernatant fluid were inoculated at different points of the comb and wattle of a white leghorn. Both the kaolin adsorbate and the supernatant fluid produced characteristic lesions, showing that the adsorption was incomplete.

Experiment 2.—(*a*) Emulsion of virus prepared as above and divided into tubes each containing 1·0 c.c. of the virus suspension. To three tubes 20 per cent. kaolin was added. One tube served as control. Those containing 20 per cent. kaolin were kept in the ice-box for 2 hours and centrifugalized. The supernatant fluid was free from protein.

The virus content of the supernatant fluid as well as that of the sediment was determined by inoculating the combs and wattles of susceptible chickens. After 8 days typical lesions developed at the points inoculated with the control virus suspension and the kaolin sediment; the points inoculated with the supernatant fluid remained negative. A 20 per cent. suspension of kaolin removed the virus without at the same time inactivating it. On the contrary the extensiveness of the lesions showed that there was a greater concentration of virus in the kaolin sediment than in the virus control.

The kaolin sediments were resuspended in 1·0 c.c. of the following eluting fluids:

(1) 2 per cent. glycocol solution in distilled water.
(2) 50 per cent. glycerine in saline.
(3) 2 per cent. glucose in distilled water.

The tubes were shaken repeatedly, left overnight in the ice-box and centrifugalized. Again the virus content of the supernatant fluid and sediment were tested as before. The sediment gave typical lesions in dilutions 1:10, but the undiluted supernatant eluting fluids gave negative results.

(*b*) This experiment was repeated using 10 per cent. kaolin suspensions as absorbant and adsorbing for 24 hours in the ice-box. The results were the same as with 20 per cent. kaolin. The virus was completely adsorbed ; the sediment produced typical lesions, while the supernatant fluid was ineffective. Elution with the solutions used in experiment (*a*) gave negative results.

Failing to obtain elution with glycocol, glucose and glycerine, we tested the effect of acids and alkalis. The following are typical experiments :

The virus suspension was adsorbed with 20 per cent. kaolin for 2 hours in the ice-box. The suspension was then centrifugalized, the supernatant fluid removed with a pipette and the same amount of the following eluting fluids added :

(*a*) 0·1 N $NH_4(OH)$.
(*b*) 0·01 N $NH_4(OH)$.
(*c*) 0·001 N $NH_4(OH)$.

The tubes were thoroughly shaken and placed in the ice-box for 24 hours. They were then centrifugalized, the supernatant fluids neutralized, and the eluate and the sediment tested for virus. The results are tabulated (Table V).

TABLE V.—*Presence of Virus in Adsorbate and Eluates.*

Eluting solution.		Sediment after elution.		Eluate.		Control.	
		Lesions.	Incubation.	Lesions.	Incubation.	Lesions.	Incubation.
NH_4OH solution	1/10 N	—	..	—	..	..	..
	1/100 N	+	6 days	—	..	..	..
	1/1000 N	++	4 „	+	6 days	..	..
Control before elution	Kaolin sediment	..	..	..	..	+	4 days
	Supernatant fluid	..	..	..	..	±	6 „

This experiment as well as several others showed that N/10 NH_4OH solution destroyed the virus ; N/100 gave variable results, depending apparently on the amount of protein buffer originally present in the virus suspension ; while N/1000 invariably eluted the virus from the kaolin adsorbate. Using N/1000 or N/500 solutions of ammonia we have been able to obtain the virus of fowl-pox in fluids free from proteins as indicated by the Esbach test. N/10,000 solutions of ammonia gave negative results, *i. e.* they failed to elute the virus, as was the case also with distilled water.

That repeated elutions with $NH_4(OH)$ yield additional virus is indicated by the following experiment :

Virus suspensions were adsorbed as above with 20 per cent. kaolin for 2 hours, centrifuged and eluted for 24 hours with N/500 and N/1000 respectively of $NH_4(OH)$. After the first eluate was removed an equal amount of fresh ammonia was added, the sediment resuspended and allowed to stand for 24 hours. The two eluates as well as the treated sediments were then tested for virus in the usual way. The results are given in Table VI.

TABLE VI.—*Extraction of Virus from Adsorbate by Repeated Elution.*

	N/500 $NH_4(OH)$.		N/1000 $NH_4(OH)$.	
	Lesion present.	Incubation time.	Lesion present.	Incubation time.
Eluate 1	+	7 days	+	7 days
,, 2	+	6 ,,	++	6 ,,
Sediment 1	+	5 ,,	+	5 ,,
,, 2	+	6 ,,	+	6 ,,

If the eluates are neutralized with phosphate solutions immediately after they are removed from the kaolin they can be kept in the ice-box for some time without losing their potency. Eluates 1 and 2, for instance, were re-tested after twenty days in the ice-box and found to have suffered no loss in potency.

The importance of the procedure is apparent. Because of their ultra-visibility and the failure to grow them in tissue-free media, the physical and biological properties of viruses have been determined in suspension of materials obtained from animal tissues or animal lesions. These suspensions contain, however, greater or lesser amounts of proteins. Consequently the results obtained as well as their variability are referable to the kind and concentration of the associated protein. That this is actually the case is demonstrated by the cataphoresis experiments reported in the next paper of this series, as well as by the experiments on the effect of disinfectants now under way. It is also to be expected that a better understanding of the immunologic phenomena of viruses will be possible by the use of protein-free virus suspensions.

CONCLUSIONS.

Experiments are reported indicating that with appropriate treatment it is possible to separate bacteriophage as well as fowl-pox virus from the associated proteins.

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STUDIES ON PROTEIN-FREE SUSPENSIONS OF VIRUSES. II.—CATAPHORESIS EXPERIMENTS WITH PROTEIN-FREE SUSPENSIONS OF A BACTERIOPHAGE AND FOWL-POX VIRUS.

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Received for publication May 14th, 1931.

IT has been shown (Kligler and Olitzki, 1931) that by adsorption with kaolin and subsequent elution with dilute ammonia solutions, it was possible to obtain bacteriophage as well as fowl-pox virus free from protein. In the present paper are presented the results of comparative cataphoresis experiments with eluted and uneluted bacteriophage and virus suspensions.

The study of the electric charge carried by bacteriophage has yielded contradictory results. According to Todd (1927) bacteriophage carries a negative charge between pH 3·2 and pH 7·7, *i. e.* within the entire range tested. Koch (1926), on the other hand, found that phage carried a positive charge and wandered to the cathode.

Similar differences are reported for viruses. Olitzki and Boez (1927) found that the virus of foot and mouth disease carries a positive charge on the acid side of pH 8·0, and a negative charge on the alkaline side. Douglas and Smith (1928) report that vaccinia virus carries a negative charge over the range of pH 5·5–8·4. Bedson and Bland (1929), using the filter-paper adsorption method, found that herpes virus was negatively charged between pH 7·6–8·0, whereas below pH 7·6 it carried a positive charge.

In our experiments we used the apparatus described by one of us (Kligler, 1931). In the case of the bacteriophage ordinary broth constituted the impure material, while the neutralized ammonia eluates (Kligler and Olitzki, 1931) served as the pure phage. In the case of the virus, suspensions of either fresh or dried crusts taken from fowl-pox lesions and the corresponding eluates were used. For comparable tests the same material was used, one part being left untreated and the other part eluted. In order to avoid any possibility of error in current, buffer solutions, etc., the tests were always run simultaneously. In the middle ranges of pH, phosphate buffers, on the acid side citrate phosphate mixtures, and in the alkaline range sodium hydroxide-glycocol solutions were used. The buffers were diluted with 0·5 per cent. saline to give N/45 to N/50 solutions. These solutions proved most satisfactory, because the resistance was sufficient to prevent an excessive flow of current, and so obviated overheating. The current was allowed to run from 2 to 3 hours—a period sufficient for marked clouding of the side arm when protein-containing suspensions were used.

CATAPHORESIS WITH PROTEIN-CONTAINING AND PROTEIN-FREE PHAGE SUSPENSIONS.

The following are the results of experiments with a coli bacteriophage :

Experiment 1.—Eluted and non-eluted bacteriophage were used. Buffer : Citrate-phosphate (after McIlvain) diluted 1 : 9 with 0·5 per cent. NaCl. Duration of cataphoresis 2 hours. The numbers represent millions of bacteriophage colonies per 1·0 c.c.

	Eluted.		Non-eluted.	
	pH 6·0.	pH 7·0.	pH 6·0.	pH 7·0.
Positive pole	16·0	36·0	20·0	28·0
Negative pole	10·0	14·0	1·0	0·0
Middle vessel	2·5	3·5	24·0	30·0
Control	10·0	20·0	20·0	32·0

Experiment 2.—Same procedure as in Exp. 1. Duration 3 hours.

	Eluted.		Non-eluted.	
	pH 4·0.	pH 5·0.	pH 4·0.	pH 5·0.
Positive pole	0·0	6·0	1·5	6·0
Negative pole	0·0	1·2	0·0	0·0
Middle vessel	0·0	0·5	40·0	40·0
Control immediately	12·0	13·5	30·0	40·0
Control after 3 hours	0·35	1·4	30·0	35·0

Experiment 3.—Phosphate buffer solutions (Sörensen) diluted 1 : 3 with 0·5 per cent. saline. Duration 2 hours.

	Eluted.		Non-eluted.	
	pH 8·0.	pH 8·6.	pH 8·0.	pH 8·6.
Positive pole	9·0	7·0	6·0	5·0
Negative pole	2·0	2·0	0·0	0·1
Middle vessel	3·5	6·0	24·0	28·0
Control	4·0	8·0	28·0	30·0

Experiment 4.—Glycocol NaOH solution diluted 1 : 5 with 0·5 per cent. saline solution. Duration of cataphoresis 3 hours.

	Eluted.		Non-eluted.	
	pH 10·0.	pH 12·0.	pH 10·0.	pH 12·0.
Positive pole	4·5	5·0	6·0	4·0
Negative pole	2·5	6·0	0·25	0·0
Middle vessel	5·0	12·0	21·0	30·0
Control	4·5	9·0	18·0	29·0

Experiment 5.—(*a*) Eluted bacteriophage (diluted 1 : 2).

	Buffer.			
	pH 5·0 McIlvain citrate phosphate (1 : 9).	pH 7·0 Sörensen phosphate (1 : 3).	pH 8·6 Sörensen phosphate (1 : 3).	pH 12·0 NaOH gycocol (1 : 5).
Positive pole	0·1	4·0	16·0	2·6
Negative pole	0·0	1·3	0·5	0·45
Middle vessel	0·6	6·5	5·0	7·5
Control	1·0	5·0	6·0	6·5
(*b*) Non-eluted bacteriophage (diluted 1 : 4).				
Positive pole	0·17	0·9	1·2	1·0
Negative pole	0·0	0·0	0·0	0·0
Middle vessel	2·5	2·5	4·0	4·5
Control	2·0	3·0	3·0	3·0

The results of these experiments demonstrate two significant points: In the protein-free suspensions the phage is highly sensitive to acid reactions and is entirely or largely destroyed at pH 4·0 and 5·0 respectively in 2–3 hours. The electric charge measured in protein-containing solutions is modified by that of the adsorbant (the protein). In protein-containing solutions the phage wanders to the positive pole at all reactions, whereas in protein-free suspensions it is amphoteric, more definitely so in acid and alkaline solutions.

CATAPHORESIS EXPERIMENTS WITH IMPURE AND PURE VIRUS SUSPENSIONS.

Having ascertained that it is possible to obtain virus suspensions free from proteins, we proceeded to duplicate the experiments made with bacteriophage. The same apparatus was used. The buffers consisted of phosphates, citrate-phosphate, or glycocol-hydroxide as required. The cataphoresis tests were made at various reactions, ranging from pH 5·0 to pH 10·0. The experiments indicated clearly that the results vary with the presence or absence of protein in the virus suspensions.

The following is a summary of typical series of experiments:

A virus suspension was prepared from fresh crusts taken from the lesions of an infected chicken. One part of the suspension was preserved in the ice-box, and the other freed from protein by absorption with kaolin and elution with N 500 $NH_4(OH)$.

Parallel cataphoresis tests were made with the original and eluted virus suspensions at pH 5·2, 7·2, 8·6 and 9·8 respectively. The duration of the cataphoresis was 2½ hours. At the end of the cataphoresis, chickens were inoculated with the original suspensions, as well as with the material from the middle vessels and positive and negative poles respectively. The results are given in Table I.

The differences in the results are of considerable interest. At pH 5·0 there is a precipitation in the protein-containing suspensions and the virus is not recoverable at either pole; in the protein-free suspension the virus is free to wander, and though amphoteric, shows a more definite tendency to move to

TABLE I.—*Results of Cataphoresis of Protein-containing and Protein-free Virus Suspensions. Inoculated December 8th, 1930.*

pH.	Date examined.	Untreated suspension.				Eluate.			
		Original suspension.	Middle vessel.	Positive pole.	Negative pole.	Original suspension.	Middle vessel.	Positive pole.	Negative pole.
5·0	14.xii	±	−	−	−	−	+	−	±
	16.xii	±	±	−	−	+	+	±	±
	19.xii	+	+	−	−	++	++	±	+
	21.xii	+	+	−	± 2 vesc.	++	++	±	+
7·2	14.xii	+	+	+	−	±	−	+	+
	16.xii	+	+	+	−	+	+	++	++
	19.xii	+	++	+	−	++	++	++	++
	21.xii	+	++	+	−	++	++	++	++
8·6	14.xii	+	−	++	−	+	−	+	−
	16.xii	++	±	++	−	+	+	+	−
	19.xii	++	±	++	−	++	++	++	±
9·8	14.xii	+	±	++	−	+	−	+	−
	16.xii	++	±	++	−	+	±	+	−
	19.xii	++	±	++	−	++	±	++	± 2 vesc.
	21.xii	++	±	++	−	++	+	++	± 2 vesc.

the cathode. At pH 7·2 the virus is definitely amphoteric in protein-free suspension, while in the protein-containing mixture this is not the case, due to the adsorption of the virus by the protein molecules. At pH 8·6 and 9·8 the virus carries a negative charge. It is apparent that the results of cataphoresis experiments with virus suspensions reported heretofore will have to be revised, since the results reported are probably due principally to the protein present. It is possible that some of the contradictory results reported in the literature have been due to variation in the amount of protein present in the virus suspensions.

The fowl-pox virus resembles the phage in its sensitiveness to acid reactions in protein-free suspensions. It differs from the phage in that it is most decidedly amphoteric in the neutral zone and negatively charged in alkaline solutions.

CONCLUSIONS.

Cataphoresis experiments with protein-containing and protein-free suspensions of a coli bacteriophage and fowl-pox virus indicate—

1. That the results heretofore reported have been partly or wholly influenced by the protein present. The charge measured was, therefore, chiefly that of the protein to which the virus was adsorbed.

2. On the basis of the results in protein-free suspensions, it appears that both the coli phage and fowl-pox virus tested are sensitive to acid reactions, and carry positive or negative charges according to the reactions of the medium.

(*a*) The phage is amphoteric in acid and decidedly alkaline solutions, and chiefly negatively charged in neutral and mildly alkaline solutions.

(*b*) The fowl-pox virus is positively charged on the acid side, amphoteric in neutral solutions, and carries a negative charge in alkaline solutions.

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QUANTITATIVE ASPECTS IN IMMUNITY REACTIONS: THE COMPOSITION OF THE PRECIPITATE IN PRECIPITIN REACTIONS.

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Received for publication May 14th, 1931.

INVESTIGATIONS of the ratio of antigen to total precipitate formed in precipitin reactions have hitherto disagreed. Wu, Cheng and Li (1928) and Wu, Sah and Li (1929) found the composition approximately constant. On the other hand, a varying composition is to be inferred from the results found by Dean and Webb (1926) and by Heidelberger and Kendall (1929), and has been actually demonstrated by Breinl and Hourowitz (1930) and Heidelberger and Kendall (1930).

In the following work we have studied the effect of variation in the ratio in which antigen and antibody were mixed on the composition of the precipitate, and the effect of non-specific proteins on the amount and composition of the precipitate.

METHODS.

Pseudoglobulin was prepared from horse-serum by precipitating three times by half saturation with ammonium sulphate and dialysis against distilled water to precipitate euglobulin. Crystalline egg albumin was prepared as described by Sörensen (1917); it was crystallized three times and dialysed against distilled water to remove ammonium sulphate.

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Azo-proteins were prepared by linking these proteins with diazotised atoxyl as described by Landsteiner and Lampl (1918), with this alteration, that 1 gm. of atoxyl, diazotised, was added to 150 c.c. of 4 per cent. protein. The coupled proteins were purified by precipitation, twice with acid and three times with alcohol; the supernatant fluid from the final precipitation was colourless. Finally they were dialysed; some colour dialysed out at first, although the dialysing sacs were impermeable to protein. Two preparations of azo-globulin, A and B, were made; A contained 1·1 per cent. and B 2·54 per cent. of arsenic; the azo-egg-albumin contained 1·53 per cent.

The absorption spectra of these preparations when dissolved in N/100 NaOH showed two bands in the ultra-violet region of the spectrum, having their maxima at 3300 Å. and 2800 Å. respectively. For reasons which appear later, the azo-protein in the precipitates studied was measured at 3600 Å. At this wave-length the azo-proteins used had the following specific extinction coefficient: A, $E = 10$; B, $E = 20$; egg albumin, $E = 11{\cdot}3$.

Rabbits were immunized by intravenous injection of 40 to 60 mgm. of azo-globulin twice a week and were first bled after the sixth injection. They remained in excellent condition throughout.

Iodo-proteins were prepared as follows: To 100 c.c. of a 4 per cent. solution of the protein were added 10 c.c. of concentrated ammonia solution (0·880), and then N/10 iodine solution, in small quantities with vigorous stirring, until free iodine could still be detected by starch at least 15 minutes after the last addition. Excess iodine was then removed by repeated addition of sodium bisulphite solution, precipitation with H_2SO_4 and resolution with NaOH (Baur and Strauss, 1929). The final solution was dialysed. The iodo-globulin contained 8·4 and the iodo-egg-albumen 6·2 per cent. of iodine.

Rabbits were immunized by three series of intravenous injections of 20–40 mgm. on three successive days with three-day intervals, and were bled five or six days after the last injection. They remained in very good condition throughout.

The anti-azo-globulin sera gave precipitates with unaltered horse pseudo-globulin. A change in the composition of the precipitate, with changing ratio of antigen to antiserum, might be attributed to precipitation occurring with different antibodies. The antiglobulin was therefore removed by precipitation by the addition of that amount of horse pseudoglobulin which gave the most voluminous precipitate as found by previous trials; usually this was 4 mgm. to 10 c.c. This fractional absorption was not done before using serum 336A with azo-egg-albumin as the precipitating antigen; nor on the serum 357A used for experiments Az. 27 to 36. The anti-iodo-protein sera gave very little precipitate with unaltered pseudo-globulin, and were therefore used without further treatment.

In all cases the optimum proportions (Dean and Webb, 1926) of antiserum and antigen were measured. The recent work of Taylor (1931) raises the suspicion that the most rapid flocculation may be the result of the balance of several factors and have no special significance; nevertheless we have found that the supernatant from the mixture in optimum proportions always became cloudy when either more antigen or more antibody was added, while the supernatants from mixtures in other proportions gave precipitates with either

antigen or antibody, but not with both. The optimum proportions could not be determined as accurately with these reactions as when untreated pseudo-globulin was the antigen.

The antibody solutions used and their optimum proportions were :

396A.	(antiglobulin removed)	1	c.c. to	1·49	mgm.	azo-globulin A.
336A.	,, ,,	1	,,	0·92	,,	,, ,,
336A.	untreated	1	,,	0·37	,,	azo-egg-albumin.
336C.	(antiglobulin removed)	1	,,	0·45	,,	azo-globulin A.
336C.	,, ,,	1	,,	0·26	,,	,, B.
357A.	untreated	1	,,	0·525	,,	,, ,,
424A.	,,	1	,,	1·87	,,	iodo-globulin.
424B.	,,	1	,,	0·23	,,	iodo-egg-albumen.
322A.	,,	1	,,	0·36	,,	iodo-globulin.
424CG.	globulin	1	,,	0·30	,,	,,
424DG.	,,	1	,,	1·7	,,	,,

To prepare the last two antibody solutions the globulin of the sera was precipitated by half saturation with ammonium sulphate, dissolved and dialysed to remove ammonium sulphate. Part of the globulin was then precipitated by bringing the sodium chloride concentration to 0·2 per cent. and adjusting the pH to 5·0. The precipitate was centrifuged off, the sodium chloride concentration of the supernatant brought up to 0·9 per cent. and the pH brought to 7·4. Of the protein in 424 DG, 28 per cent. was precipitated with the antigen in experiment Io. 18.

In all the experiments, except those on the effect of non-specific proteins, also Az. 35 and 36, and experiments T.A.T. and G.A.G., the antigen in as strong a solution as could be measured satisfactorily was added, with stirring, to the undiluted serum. The mixture was frozen solid and left frozen in the ice-chest overnight, then thawed, centrifuged, and washed twice with 0·9 per cent. NaCl solution to which had been added one-tenth its volume of M/15 phosphate mixture, giving a pH of approximately 6·7.

In the experiments shown in Table III the sera were diluted with 0·9 per cent. NaCl or with normal rabbit serum. The mixtures were stood for 3 hours at room temperature and kept in the ice-chest overnight.

The precipitates formed with azo-protein as the antigen were dissolved in N 100 NaOH, and the amount of azo-protein present estimated spectrometrically at 3600 Å. This wave-length was chosen instead of the maximum at 3300 Å., because at the latter wave-length there is a risk of some interference by the ultra-violet absorption of other protein present (Smith, 1929; Smith and Marrack, 1930); at 3600 Å., however, only the azoprotein is measured. Over the range of concentrations measured Beer's law was found to be obeyed whether other protein was present or not. The absorption was measured by a photo-electric method (Smith and Holliday, to be published).

The precipitates formed with iodo-proteins as antigens were dissolved in N/500 NaOH. Iodine was estimated by incineration of an amount of solution, containing about 0·010 mgm. of iodine and 1 mgm. of protein, with about 0·2 gm. of K_2CO_3. The iodide formed was extracted with alcohol, oxidised with bromine water and titrated with N/100 potassium thiosulphate; all

estimations were made in duplicate. These estimations did not present the difficulties usual in estimating iodine in biological material, as the ratio of iodine to organic matter was relatively large. It was found that when iodine was added as potassium iodide to 1 mgm. of protein, 95 per cent. could be recovered. The duplicates agreed within 5 per cent.

TABLE I.—*Azo-proteins.*

Antiserum.	Precipitating antigen.	A/G.	Precipitate from 1 c.c antiserum. Total N, mgm.	Antigen N, mgm.	$\frac{\text{Antigen N}}{\text{Total N}} \times 100$.
396A.	Azo-globulin A.	6/1	0·105	0·025	24
,,	,,	4/1	0·15	0·045	29·5
,,	,,	1/1	0·39	0·154	39
,,	,,	1/3	0·59	0·312	53
,,	,,	1/5	0·655	0·380	58
,,	,,	1/9	0·335	0·230	68
336A.	,,	2/1	0·19	0·058	31
,,	,,	1/1	0·30	0·110	37
,,	,,	1/3	0·37	0·202	55
,,	,,	1/6	0·45	0·270	60
336C.	Azo-globulin B.	3/1	0·077	0·0064	9
,,	,,	1/1	0·175	0·0185	10·5
,,	,,	1/3	0·295	0·0395	13
,,	,,	1/6·4	0·32	0·062	19·5
,,	,,	1/7·3	0·27	0·066	24·5
,,	Azo-globulin A.	1/1	0·20	0·023	11·5
,,	,,	1/7	0·29	0·073	25·5
336A.	Azo-egg-albumin	2·2/1	0·084	0·014	17
,,	,,	1/1	0·14	0·032	22·5
,,	,,	1/3	0·195	0·0615	38
,,	,,	1/6	0·22	0·100	45

TABLE II.—*Iodo-proteins.*

Antiserum.	Precipitating antigen.	A/G.	Precipitate from 1 c.c. antiserum. Total N, mgm.	Antigen N, mgm.	$\frac{\text{Antigen N}}{\text{Total N}} \times 100$.
424A.	Iodo-globulin	2·5/1	0·232	0·0315	13·5
,,	,,	1/1	0·425	0·066	15·5
,,	,,	1/1·8	0·485	0·106	22
,,	,,	1/3·3	0·485	0·129	26·5
,,	,,	1/5·4	0·400	0·121	30
322A.	,,	3/1	0·085	0·009	10·5
,,	,,	1/1	0·145	0·019	13
,,	,,	1/3	0·140	0·027	19
,,	,,	1/5	0·086*	0·015*	17·5*
424B.	Iodo-egg-albumin	3/1	0·071	0·007	10
,,	,,	1/1	0·137	0·024	18
,,	,,	1/3	0·170	0·0375	22
,,	,,	1/5	0·085	0·0235	27·5

* These figures are less accurate than the rest, as the amounts available for analysis were small.

The amount of iodo-protein present was calculated from the amount of iodine found.

In the tables A/G stands for the ratio in which antigen and antibody were mixed, and is given as 1/1 when they were mixed in optimum proportions.

RESULTS.

The experiments in Tables I and II show that with increasing ratio of antigen to antibody, (1) the total amount of precipitate obtained from a given amount of antiserum rises to a maximum when the antigen is in moderate excess and then falls off, and (2) the percentage of antigen in the precipitate rises and may even be nearly trebled. This change of composition was obvious on mere inspection of the colour and consistency of the precipitate.

It also appears that when a weaker serum was used, the amount of precipitate from a given amount of serum and the percentage of antigen in the precipitate was less. With the weak serum 322A, the variation in the composition of the precipitate was less than with the stronger serum 424A.

Although azo-globulin A contained less arsenic and presumably fewer combining groups than B, the precipitate that is formed with a given amount of serum was approximately the same in amount and composition.

TABLE III.—*Effect of Non-specific Protein.*

Experiment No.	Antiserum.	Precipitating antigen.	A/G.	Precipitate from 1 c.c. antiserum.			Protein in supernatant %.	Dilution of 1 c.c. antiserum with—
				Total N, mgm.	Antigen N, mgm.	Antigen N / Total N × 100.		
Az. 27	357A.	Azo-globulin B.	1/1	0·282	0·0435	15·4	1·8	2·5 c.c. saline.
Az. 28	,,	,,	,,	0·287	0·0447	15·6	5·45	2·5 ,, normal serum.
Az. 31	,,	,,	1/5	0·320	0·0665	20·8	1·9	2·5 ,, saline.
Az. 32	,,	,,	,,	0·445	0·0895	20·1	5·6	2·5 ,, normal serum.
Az. 33	,,	,,	3/1	0·150	0·0126	8·4	1·8	2·5 ,, saline.
Az. 34	,,	,,	,,	0·152	0·0129	8·5	5·4	2·5 ,, normal serum.
Io. 18	424D.G.	Iodo-globulin	1/1	1·23	0·136	11·1	0·45	2·0 ,, saline.
Io. 19	,,	,,	,,	1·57	0·163	10·4	3·9	2·0 ,, normal serum.
Io. 20	424C.G.	,,	,,	0·071	0·011	15·5	0·09	1·0 ,, saline.
Io. 21	,,	,,	,,	0·095	0·0135	14·2	2·9	1·0 ,, normal serum.

The experiments in Table III show the effect of diluting the antiserum with normal serum (Experiments Az. 28, 32, 34, 19, 21) instead of normal saline (Experiments Az. 27, 31, 33, 18 and 20). They show that although the amount of precipitate may be increased by the addition of non-specific protein, the composition is little changed. This result can be most simply explained by supposing that the antigen-antibody compound is more completely precipitated in the presence of non-specific protein, a small quantity of which may be adsorbed and produce a slight change in the ratio of antigen to total protein in the precipitate. With the azo-proteins this effect was only appreciable when

antigen was in excess (*cf.* Dean, 1912, and the effect of $BaCl_2$, Marrack and Smith, 1931).

We also found that the time of flocculation was usually shortened by the addition of non-specific protein, and that this was most conspicuous when antigen was in excess; in Experiment Az. 31 flocculation took place in two hours, in Az. 32 in one.

A point, the importance of which will appear in the discussion, is illustrated by experiment Az. 29. This was done exactly as experiment 28, except that the normal serum was replaced by an equal amount of antiserum 357. The total precipitate nitrogen was much increased, being 0·71 mgm., and the antigen nitrogen formed only 8·25 per cent. of the total; flocculation took place at once on mixing in this experiment, and in 20 minutes in experiment 28. The effect of additional antiserum is therefore very much greater than that of normal serum.

The following experiments, Az. 35 and 36, show that the increase of protein in a precipitate when excess of antibody is added is accompanied by an uptake of antibody. Assuming that the precipitate A in Az. 36 had the same composition as that from the similar mixture in 35, we can calculate that the extra protein nitrogen taken up from the additional 2 c.c. of antiserum in the second stage was 0·21 mgm. If we assume that this protein is antibody, it is possible to calculate the proportion of antibody which has been taken up by the precipitate. From the composition of the precipitate in Experiments Az. 27 and 28 we see that 0·21 mgm. of protein nitrogen combines with 0·038 mgm. of dye nitrogen at optimum proportions. But we also know from the method of optimum proportions that 0·070 mgm. of dye nitrogen is equivalent to 1 c.c. of antiserum, so that 0·038 mgm. is equivalent to 0·54 c.c. of antiserum. Therefore some 27 per cent. of the 2 c.c. of antibody added in the second stage was taken up, which is approximately what was found by the optimum proportions titration.

Experiment Az. 35.—To 1 c.c. of serum 357A was added 0·1 c.c. of a solution of azo-globulin B, containing 0·35 mgm. N.; the A/G ratio was 1/5. The mixture was left at room temperature for 5 hours and in the ice-chest overnight. The washed precipitate contained total N 0·387 mgm., antigen N 0·071 mgm. and therefore non-antigen N 0·316 mgm.

Experiment Az. 36.—To 0·5 c.c. of serum 357A was added 0·05 c.c. of the solution of azoglobulin B, containing 0·175 mgm. N, and the mixture treated as in Experiment 35. The precipitate so obtained (precipitate A) was rubbed up with a further 2 c.c. of the antiserum 357A, and the suspension kept for 5 hours at room temperature with repeated shaking and over night in the ice-chest. The precipitate centrifuged off and washed contained total N 0·40 mgm., antigen N 0·035 mgm., and therefore non-antigen N 0·365. Assuming that the composition of precipitate A was the same as that in experiment 35, the extra protein taken up from the further 2 c.c. of antiserum was 0·21 mgm.

The optimum proportions for the supernatant fluid were found to be 1 c.c. of fluid to 0·047 mgm. of azo-globulin, while that for the original serum was 1 c.c. to 0·070 mgm.

In experiments T.A.T. and G.A.G., in which a precipitation was carried out in the presence of a non-homologous protein antigen, which can be detected,

this protein was not carried down. These experiments exclude the theory that the increase in amount of antigen found in the precipitate with such antigens as azo-globulin, when increasing amounts of antigen are used, is due to non-specific adsorption.

Experiment T.A.T.—12·5 c.c. of diphtheria toxin were mixed with 2 c.c. of a 3·7 per cent. azo-globulin solution. The equivalent amount of antitoxin, 0·073 c.c., determined by the flocculation method, was then added, and the mixture kept for 3 hours at 45° C. and overnight in the ice-chest. The concentration of azo-globulin in this mixture was as high as in a mixture of antiserum 336A and antigen with the A/G ratio 1/6. The precipitate formed, which contained 0·36 mgm. of nitrogen, dissolved in N/100 NaOH to form a completely colourless solution. No azo-globulin, therefore, was carried down with the toxin-antitoxin precipitate.

Experiment G.A.G.—1 c.c. of an anti-pseudoglobulin serum was mixed with 2 c.c. of 0·9 per cent. saline and 0·1 c.c. of a 3·05 per cent. solution of azo-egg-albumin. Then one-third the optimum proportion of horse pseudo-globulin was added. The mixture was kept 5 hours at room temperature and in the ice-chest overnight. The precipitate dissolved in N/100 NaOH was completely colourless. No azo-globulin was therefore carried down by this precipitate, which contained excess of antibody.

DISCUSSION.

We agree with Breinl and Hourowitz (1930) and Heidelberger and Kendall (1930) in finding an increase in the percentage of antigen in the precipitate when increasing amounts of antigen were added to a given amount of antibody.

The disagreement between Breinl and Hourowitz and Wu, Cheng and Li (1928) in the results with hæmoglobin as antigen can be explained, since Breinl and Hourowitz found the composition fairly constant over a wider range than that over which Wu, Cheng and Li worked. Our disagreement with Wu, Sah and Li (1929) in our results with iodoproteins can be explained by the fact that their sera were weaker than our serum 424 and that they worked over a narrower range ; with one of their strongest sera the composition of the precipitate varied appreciably.

Heidelberger and Kendall (1929) suggest that precipitates may consist of mixtures of two compounds, AG and AG_2, where A and G are antibody and antigen molecules. This is not possible if the laws of mass action are applicable to the reaction (which may not be the case, as the reactions may be partly irreversible). It is probable that the gradual change in the composition of the precipitate is due to the formation of compounds of the formula A_mG_n ; such compounds will be formed if antibody and antigen molecules have several combining groups.

The relation of the amount and composition of the precipitate to the strength of the serum is what might be expected, if the strength of the serum were dependent on the amount of antibody globulin it contained and the number of binding groups per molecule of this antibody. Similar results were found by Dean and Webb (1926) with serum as antigen, and by ourselves (1930) with diphtheria toxin and antitoxin.

The difference in the effects of non-specific protein found in these experiments and in those with pseudoglobulin (1931) and diphtheria antitoxin (1930) is probably due to the fact that the sera were less potent, and the denaturation of the antibody globulin less complete. It cannot be overlooked that the last stage in a precipitin reaction is the flocculation of a more or less lyophobe sol. It is to be expected that the presence of non-specific proteins, especially euglobulin, may affect the rate and completeness of this flocculation, and that a small quantity of this foreign protein may be absorbed. The fact that in some cases the amount of precipitate is unaffected by extreme variations of non-specific protein, is strong evidence that the protein in the precipitate plays an essential part in the reaction and is not merely adsorbed ; while a variation in the amount, and even a slight variation in the composition of the precipitate in the presence, of additional non-specific protein, does disprove the idea that the main part of the protein attached to the antigen in the precipitate is actually antibody.

Taylor (1931), to explain the results of titrations of the supernatant fluids from precipitin reactions and the effect of non-specific serum on the rate of flocculation, suggests that the antigen-antibody compound may adsorb from serum a non-specific substance, which again may adsorb excess antigen or antibody. His titrations, by the optimum proportions method of the supernatant fluid, indicate that the amount of antibody bound by a given amount of antigen is not increased when antibody is added in excess, and he would presumably consider the increase in the ratio of total protein to antigen in the precipitate that we and others have found to be due to this adsorption of this non-specific substance. We consider it probable, however, that the application of the optimum proportions method to the supernatant may not be sound, owing to the remains of the serum and altered antigen and antibody present ; and our Experiment 35, in which an optimum proportions titration undisturbed by residues of a previous precipitation is used, shows that antibody approximately proportional to the additional protein is taken up.

As our Experiments Az. 28 and 29 show, the effect of antiserum is of a different order to that of normal serum, and the effects of excess antiserum cannot be accounted for merely by a hypothetical non-specific substance in it.

Experiments T.A.T. and G.A.G. show that the union of antigen-antibody compound with further antigen is specific ; the compound will not take up *any* substance that will act as an antigen, but only one related to that used for immunization. In the same way the union with further antibody is specific, this fact being the basis of the method of fractional adsorption (see also Landsteiner and Prasek, 1911). Such a specific combination does not indicate adsorption by a non-specific substance from serum, but a further specific combination of antigen with the antibody in the complex, or *vice versâ*.

CONCLUSIONS.

1. The ratio of antigen to total protein in the precipitate formed in a precipitin reaction increases when increasing amounts of antigen are added to a given amount of antibody.

2. The union of the antigen-antibody compound with more antigen or antibody, when either is added in excess, is not due to non-specific adsorption.

3. The total amount of precipitate from a given amount of serum, and the ratio of antigen to total protein, are higher with strong sera than with weak sera.

4. The amount of precipitate and rate of flocculation may be increased by the addition of non-specific proteins, but the ratio of antigen to total protein in the precipitate is little altered.

5. It is inferred that non-specific proteins may increase the rapidity and completeness of flocculation of the antigen-antibody compound, but are very little adsorbed.

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EFFECTS OF HÆMORRHAGE ON THE VASCULAR NERVOUS MECHANISM.

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Received for publication April 24th, 1931.

THAT alteration of the excitability of the bulbar centres should occur as a result of variations in the amount of the circulating blood is an idea which naturally presented itself to many workers. Stewart and co-workers (1906), Gesell (1918), Bayliss (1918), Richet (1918), Henderson and Haggard (1922) have all investigated different aspects of the problem.

Barry (1923) showed the influence of definite degrees of hæmoglobin privation, and of pressure change on the excitability of three bulbar centres.

RHEOBASE AND CHRONAXIE.

In light of the work which has been done in recent years concerning liminal intensity and duration of stimulation, and considering the proved importance of rheobase and chronaxie as parameters of excitability, it seemed to the present authors to be highly desirable to determine the influence of hæmorrhage on these factors in central and peripheral vagal responses and in vasomotor reactions.

Tests of excitability were previously made as a rule with the induction coil, but there is little similarity between the threshold values obtained from this and those from the galvanic current expressed in terms of intensity and duration.

The rheboasic factor—that is, the liminal effective voltage of a galvanic current applied during infinite time—represents a part of the total quantity of excitation necessary; therefore it gives a measurement of excitability; and the chronaxie or minimum effective duration of a current of twice rheobasic strength gives another. The chronaxic values are given in this work in milli-microfarads of condenser capacity, which is a function of time of discharge and therefore expresses duration.

METHOD OF INVESTIGATION.

The method employed has been already described (Barry and Chauchard, 1930), but a few further details are essential. A carotid artery was used for bleeding and a femoral for blood-pressure record. Blood was restored after defibrination through the jugular vein. One vagus nerve was cut for testing central inhibition, both for V.M. reaction.

Non-polarizable electrodes were applied to the nerve in the usual way, the trunk being enclosed in a cork tube over a certain distance and carefully protected from exposure. The electrodes were connected to a circuit for iterative or repeated excitations, which, of course, are essential for the vagus nerve, by charge and discharge of condensers of different capacity.

In many of the experiments natural respiration was not interfered with; the influence of vagal respiratory reactions on cardio-inhibitory responses, for instance, does not matter. But in others artificial respiration was employed so as to obviate the influence on the vascular system of respiratory modifications. This was especially done in tests of vaso-motor reactions.

The technique for stimulation was similar to that previously described for investigating the effects of mitral insufficiency on vagal chronaxie, in which was first determined the value of the threshold excitation for inhibition in the normal condition, and then its value for the same response after a lesion of the mitral valve had been set up by the withdrawal of a plunger from a tube within the heart. Similarly in the present work, the values of rheobase and chronaxie being ascertained for normal conditions, they were further determined after different degrees of bleeding, and finally after reinjection of the withdrawn blood. The bulbar centres never being subjected to the influence of too prolonged or too severe anæmia, one could generally count on a recovery of function in them on restoring the circulation.

The figures for current strength for summation in function of interval between stimuli were also determined for each change, and the laws thus

established are given at the bottom of the tables showing the main effects. These summation curves are significant in showing certain changes of ascent or descent which do not always correspond to the simple chronaxic variation. The intervals between stimuli vary from $\frac{1}{1\,2}$ of a second to $\frac{1}{3}$ of a second, and the increase of voltage necessary to produce stimulation with each increase of interval is noted.

RESULTS.

The general nature of the results may be seen from some of the protocols of the experiments set out in Tables I to IV.

TABLE I.—*Reflex Vaso-motor Effect; Moderate Bleeding. Dog, 9 Kilos.*

Anæsthetic.	Vagus excited.	Other vagus.	Arterial pressure in mm. Hg.	Amount bled in c.c.	Amount infused.	Reaction.	Rheobase, volts.	Chronaxic capacity in m.mf.
$ChCl_3$ and morphine	Right C. E.	Cut	95	..	..	Rise of B.-P.	10	120
Ditto	Ditto	,,	30	450	..	Ditto	10	400
,,	,,	,,	45	..	..	,,	8	450
,,	,,	,,	65	..	300	,,	15	150
,,	,,	,,	70	..	..	,,	10	140

A. Law of intervals at first (95 mm. Hg.)	Intervals . . .	$\frac{1}{1\,2}$	$\frac{1}{8}$	$\frac{1}{6}$	$\frac{1}{4}$
	Voltages . . .	20 (rh. × 2)	22	25	32
B. Law of intervals at 30 mg. Hg.	Intervals . . .	$\frac{1}{1\,2}$	$\frac{1}{8}$	$\frac{1}{6}$	$\frac{1}{4}$
	Voltages . . .	16 (rh. × 2)	25	29	42
C. Law of intervals on recovery	Intervals . . .	$\frac{1}{1\,2}$	$\frac{1}{8}$	$\frac{1}{6}$	$\frac{1}{4}$
	Voltages . . .	20 (rh. × 2)	21	21·5	23

Remarks.—In this experiment the variations of the rheobase are unimportant except for the figure 15 at 65 mm. Hg. The chronaxie change is typical. The curve of intervals is discussed in the text; the change in it is fairly typical.

TABLE II.—*Reflex Vaso-motor Effect; Rapid Severe Bleeding. Dog, 15 Kilos.*

Anæsthetic.	Vagus excited.	Other vagus.	Arterial pressure in mm. Hg.	Amount bled in c.c.	Amount infused.	Reaction.	Rheobase, volts.	Chronaxic capacity in m.mf.
$CHCl_3$ and morphine	Right C. E.	Cut	110	..	..	Rise of B. P.	10	350
Ditto	Ditto	,,	40	700	..	Ditto	15	1000
,,	,,	,,	35	100	..	,,	40	1200
,,	,,	,,	100	..	800	,,	8	300

Remarks.—In this example both rheobase and chronaxie much increased after hæmorrhage, and both quickly restored to initial value with normal circulation.

TABLE III.—*Peripheral Inhibition; Severe Bleeding. Dog, 19 Kilos.*

Anæsthetic.	Vagus excited.	Other vagus.	Arterial pressure in mm. Hg.	Amount bled in c.c.	Amount infused.	Reaction.	Rheobase, volts.	Chronaxic capacity in m.mf.
$CHCl_3$ and morphine	Right P. E.	Cut	90	..	..	Fall of pressure	9	80
Ditto	Ditto	,,	20	1000	..	Ditto	35	300

A. Law at outset	Intervals . .	$\frac{1}{12}$	$\frac{1}{8}$	$\frac{1}{6}$	$\frac{1}{4}$	$\frac{1}{3}$
	Voltages . .	18 (rh. × 2)	21	22	26	32
B. Law at 20 mm. Hg.	Intervals . .	$\frac{1}{12}$	$\frac{1}{8}$	$\frac{1}{6}$	..	..
	Voltages . .	70 (rh. × 2)	70	90	..	..

Remarks.—This is an instance of rapid severe bleeding. Rheobase and chronaxie both quickly increased. Recovery of original rheobase and chronaxie not completed on reinjection, but much diminished.

TABLE IV.—*Peripheral Stimulation; Slow Bleeding. Dog, 12·5 Kilos.*

Anæsthetic.	Vagus excited.	Other vagus.	Arterial pressure in mm. Hg.	Amount bled in c.c.	Amount infused.	Reaction.	Rheobase, volts.	Chronaxic capacity in m.mf.
Chloralosane	Right P. E.	Intact	100	..	..	Fall of pressure	4	120
Ditto	Ditto	,,	60	250	..	Ditto	5	200
,,	,,	,,	30–40	200	..	,,	5	250
,,	,,	,,	15	200	..	Rise of pressure	4	70
,,	,,	,,	15	..	..	Fall of pressure	6	250
,,	,,	,,	60	..	350 gum saline	Ditto	4	150

A. Law of intervals at outset	Intervals .	$\frac{1}{12}$	$\frac{1}{8}$	$\frac{1}{6}$	$\frac{1}{4}$	$\frac{1}{3}$
	Voltages .	8 (rh. × 2)	9·5	12	20	24
B. Law at 60 mm. Hg. (after first bleeding)	Voltages .	10	24	30	50	..
C. Law at 60 mm. Hg. (after saline injection)	Voltages .	8	12	17	34	..

Remarks.—In this experiment an anomalous result was obtained at low arterial pressure, viz. a rise of pressure following vagal excitation at low chronaxie (70 m.mf.), but with voltage 6 in the same condition the normal reaction was obtained with high chronaxic capacity.

In the experiment described in Table I the rheobase and chronaxie of the vaso-motor reaction are respectively 10 volts and 120 m.mf. in normal conditions with an arterial pressure of 95 mm. Hg. After bleeding to the extent of 450 c.c., or about 70 per cent. of the calculated total blood, the rheobase is at first unchanged, while the chronaxie is nearly quadrupled. On re-introduction of 300 c.c. of the withdrawn blood the rheobase at first rises, but the chronaxie falls, though not to the original value. The experiment provides an instance of opposite variations of rheobase and chronaxie, which will be dealt with in the discussion which follows. The alteration of the chronaxie follows the general rule observed for this factor.

The curves expressing the changes in the law of intervals in this experiment show that the increment of voltage with increase of interval is, after loss of blood, out of proportion to that observed before bleeding, and that the re-introduction of blood causes a marked flattening of the curve with small increments of voltage.

In Experiment 2 is seen an instance of an initial high chronaxic value. The effect of a rapid severe bleeding was a sharp increase of both rheobase and chronaxie, which became normal after re-introduction of blood.

The illustrations (Figs. 1 to 4) show the type of record obtained in an experiment to determine changes of the central cardio-inhibitory reaction. Numerous excitations have to be made with changing rheobase and chronaxie until a steady response is obtained, and it is not easy to confine salient features within a limited space on the recording surface. In the record (Fig. 1) are seen the responses taken as normal thresholds, viz. chronaxie 120 m.mf. (at third cross mark) and rheobase 15 (at last +). Each stimulation is marked by a cross in all records, and the respiratory tracing is placed below that of blood-pressure. Three double records are seen in Fig. 2. The central one shows the final test of chronaxic value at 120, just before bleeding, which is seen to be accompanied by a fall of pressure. Some 30 p.c. of the calculated total of blood was withdrawn, and the blood-pressure fell by about 35 mm. Hg. The bottom record is a continuation at low pressure of the central one. It shows the chronaxic response at 300 m.mf. which before bleeding was 120. At the second mark it is seen to be insufficient with 250 m.mf. The last stimulation is for the rheobase, which in this case was slightly less than before. The top portion of the record shows ineffectual intermediate stimulation to ascertain the new chronaxie. On reintroduction of the withdrawn blood in this experiment (Fig. 3) the amplitude of the beats became larger, but the chronaxie, which was later restored to something near its original value, remained high for some time. It will be observed that a capacity of 300 (second stimulation) is essential to elicit a reaction which is comparable to that previously obtained. A second bleeding (Fig. 4) was performed after restoration, and several stimuli reveal changes which are much the same as with the first bleeding.

Slight differences may be noted in the degree of the responses which are taken as threshold ones. Absolute correspondence between them was not sought for; it causes too much delay, which of course involves adaptations slowly established and disturbance of the picture of immediate effect. There is not much variation of a particular change of reaction within five or ten minutes of its occurrence, but after that interval there are changes which

constitute a somewhat different problem from that on which we are engaged here.

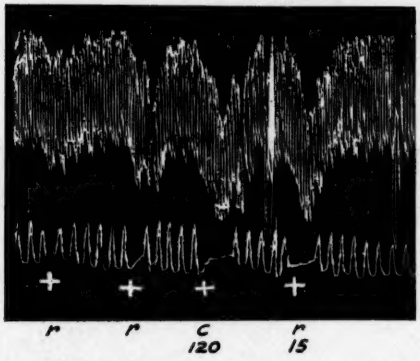

FIG. 1.—Threshold reaction. Chronaxie and rheobase.

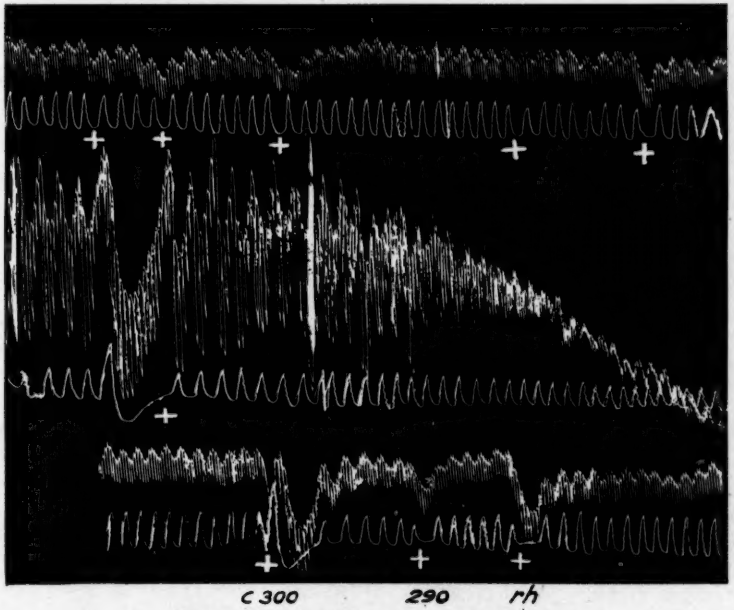

FIG. 2.—Three double records of B.P., and R.

Thus hæmorrhage always involves an increase of the chronaxie in the vasomotor and cardio-inhibitory centres. A reversal of the reaction of the former centre—that is, depressor instead of pressor response—was obtained in one

instance as a result of bleeding. We have not as yet been able to dissociate these two responses, constrictor and dilator, by differences of the time factor in the application of an afferent stimulus.

The influence of loss of blood on the peripheral mechanism of the cardiac vagus is also to cause an increase of the chronaxie. In Experiment 4 a series of moderate bleedings resulted in increase from 120 to 200 and 250 m.mf., and a slight increase of the rheobase. An anomalous effect was observed in this

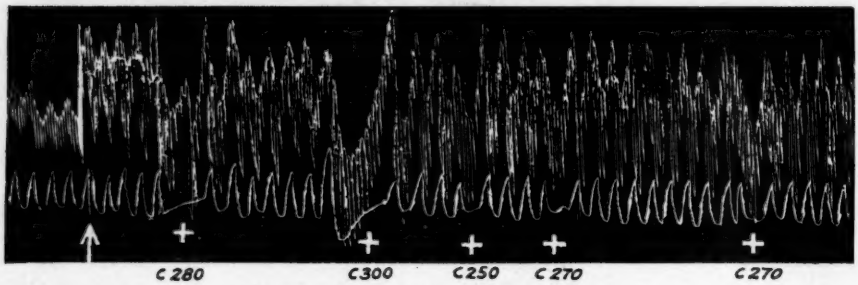

FIG. 3—Reintroduction of blood, at arrow.

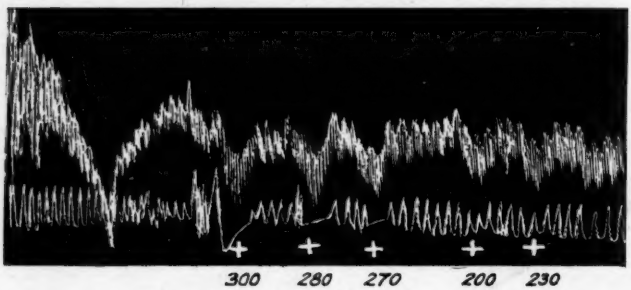

FIG. 4.—Second bleeding.

condition when with small voltage and chronaxic value (70) a rise of blood-pressure followed peripheral stimulation, the second vagus being intact. On increasing both values to 6 and 250 respectively the inhibitory reaction returned.

In Experiment 3 a rapid severe hæmorrhage determined a marked rise both of rheobase and chronaxie—an effect which has also been observed in other instances. In this case summation at an interval of 1/6 needed a sharp rise of voltage, and no available voltage was sufficient for summation at 1/4.

DISCUSSION.

The regularity with which loss of blood causes increase in the chronaxie of medullary centres and peripheral end-organs is very striking. *À priori*, one might expect a difference in the new modalities of excitation as between

vaso-motor and cardio-inhibitor excitability. Increase of chronaxie means decrease of excitability, and in a centre which is called on to combat a fall of blood-pressure an increase of excitability as occasioned by this fall would not be very surprising. However, the result demonstrates uniformity of susceptibility of the elements to changes of pressure and volume flow.

How much of the change is due to modification in the centre and how much to alteration in effector organ it is not always possible to say. A purely cardiac change, such as that caused by experimental mitral insufficiency, has been shown to determine an alteration both of peripheral and central chronaxie of the inhibitory response. Or rather, the cardiac response was altered, whether elicited by direct peripheral stimulation or by central reflex stimulation. Also in case of hæmorrhage a double mechanism is concerned, but we don't know if both parts are equally affected, or if the reaction of the peripheral organ alone is modified.

Anæmia of the centre is one factor, anæmia of the heart, or a lowered coronary pressure, is another. Central and vascular factors have also to be taken into account for the vaso-motor reaction.

That the effect is not solely due to the diminution of oxygen is indicated in Experiment 4, where injection of saline solution by restoration of pressure and volume flow caused a diminution of augmented chronaxie. In some instances it was found that saline injection fully restored the initial chronaxie, but unlike what happened with re-injection of blood, it had not a lasting effect in this respect.

We have observed certain discrepancies between rheobase and chronaxie in these experiments which are difficult to account for. In some instances a diminution of the former is accompanied by an increase of the latter. The rate of bleeding seems to have more influence on the rheobasic than on the chronaxic change, the liminal voltage being as a rule definitely increased with rapid bleeding, whereas we have seen it diminished in case of gradual loss. This seems to suggest that for a certain type of variation of excitability the intensity of the stimulus necessary is less while its duration must be increased. We must, however, take this intepretation of a new modality with reserve. Some slight change in the position or surroundings of the electrodes may have a marked influence on rheobasic value, and rapid variations of the water content of the tissues cause changes of resistance between them. Some of the anomalies observed were described to L. Lapicque and his comments asked for. He considers that quantitative estimates of excitation in terms of rheobase and chronaxie need further investigation, which he is about to undertake, but he is of opinion that all values which are carefully observed and controlled might be published at the present juncture. Whatever be the values of the rheobase, the chronaxic figures are valid for experiments of this sort.

The curves of summation or voltage in function of interval rise as a rule after hæmorrhage, but in some instances a tendency to flattening was observed. The increments of voltage necessary for summation with each increase of interval seem to depend to some extent on the rapidity and degree of bleeding, and there are certain differences according to the type of reaction. A flattening of the curve, expressing a diminution of voltage increment necessary for each increase of interval, was observed for central inhibitory reactions. The

except in case of severe and rapid hæmorrhage, when it is markedly increased significance of the variations in the laws of summation will be clearer when proper quantitative values are ascertained for the different proportions of rheobase and chronaxie.

An interesting example of different chronaxies of stimulus eliciting different reactions in the same mechanism is provided in Experiment 4. When the chronaxic capacity used for excitation of the peripheral vagus was 70—that is, lower than the initial normal value for inhibition—an anomalous effect of rise of pressure was obtained. But with increased voltage and a capacity of 250 (which had been found as the new anæmic chronaxie of inhibition) the ordinary inhibitory response returned. This was not a mere chance observation, as it was repeated a few times. The unstimulated vagus in this case was intact, so that the chronaxie of the organ should rightly be described as that of subordination (Lapicque). This second vagus was not subsequently severed, as we desired to maintain uniform conditions to the end of the experiment, but it is possible that section of it would do away with the anomaly. The point is worthy of further investigation, which we may be in a position to carry out at some future date.

The restoration of the normal time-factor in the excitability of the different mechanisms by restoration of the ordinary conditions of the circulation shows that the property of excitability, so far as duration of effective stimulation is concerned, is a definite function of the condition of the circulation. While ultimately it must be a function of hæmoglobin content and oxygen supply, we have seen that it is closely dependent on the pressure and volume flow to which the excitable elements are exposed. This is obvious from the temporary return of the original properties on substitution of saline solution for blood.

The close dependence of the excitability of these nervous elements on circulatory conditions forms a contrast with what we observe in an excised nerve muscle preparation, in which the chronaxie has much the same value as that of the muscle *in situ* with free circulation. In the case of the muscle *in situ*, however, where the central nervous connections are maintained, we are dealing with the chronaxie of subordination—a variable property as distinct from the constitutional chronaxie of the isolated muscle, which is fixed, and which remains the same as long as the muscle is irritable. We do not know if any influence of circulatory changes on the chronaxie of subordination has been shown to exist for skeletal muscle, but it seems not unlikely that it should. In the cardiac reaction we have been dealing with the chronaxie of subordination. The constitutional chronaxie would come into play only if both vagi and sympathetics were cut. One may count on it in the heart-lung preparation. With this latter we have made a few tests of vagal chronaxie with a view of determining changes in it during the course of survival, but without specially altering the amount of blood or the coronary pressure, the influence of which we hope to determine later on.

SUMMARY AND CONCLUSIONS.

Modifications of the excitability of the parasympathetic inhibitory mechanism of the heart and of the vaso-motor mechanism are shown to occur as a result of hæmorrhage in dogs. Changes of the rheobase are not distinctive

in value, and this holds for both peripheral and central stimulation of the inhibitory apparatus and for central stimulation of the vaso-motor.

The chronaxie, on the other hand, is invariably increased by loss of blood for all the reactions tested, and the extent of the increase is roughly proportional to the severity of the hæmorrhage. Restoration of the blood lost, after defibrination, causes more or less complete return to former values of excitability. Temporary recovery of these values is also determined by injection of saline solution.

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A STUDY OF CROSS-IMMUNITY WITH VIRUSES OF FOWL-PLAGUE AND OBSERVATIONS ON THE DURATION OF IMMUNITY.

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Received for publication May 19th, 1931.

IT is known that in certain diseases due to filterable viruses, such as foot and mouth disease and swine fever (East African and European strains), more than one immunological type of virus exists.

In the case of fowl-plague, however, the work of Todd (1928) has shown that birds immunized with an English strain of virus (Rice) are immune to Dutch (de Blieck), French (Staub) and German (Miessner) strains.

This investigation was undertaken to verify and extend to viruses of non-European origin Todd's conception of the immunological unity of fowl-plague virus.

CROSS-IMMUNITY TESTS WITH VIRUSES OBTAINED FROM DIFFERENT LOCALITIES.

A number of strains of fowl-plague virus from different sources were collected and their immunological relations studied. The following list shows the viruses used, and their sources :

(1) The " Rice " strain is that isolated by Rice (1924).

(2) The " Bax." strain was isolated in 1929 at this laboratory by Mr. N. Hole.

(3) The "Ex." strain was also isolated by Mr. N. Hole, but from a different outbreak.

(4) The Palestine strain was obtained through the kindness of Mr. J. Smith, C.V.O., Palestine.

(5) The Egyptian strain was obtained from M. Lagrange.

(6) The French strain is that of Staub.

(7) The Dutch strain is that of de Blieck.

Both the French and Dutch strains were procured by Mr. T. M. Doyle, of this Laboratory.

Immunizing the Fowls.

It was found that to obtain fowls immune to the various strains of fowl-plague viruses was a very difficult undertaking. A number of birds were immunized by Todd's method, and the results obtained with this method have been reported in a previous paper (Purchase, 1930).

A few of the fowls used were immunized during the course of experiments dealing with the optimum pH for survival of the virus. On a number of occasions, in these investigations, the inoculated birds did not die of fowl-plague, and were found to be immune on subsequent testing with virulent material. Although the procedure was repeated, the results were very inconsistent, as only a proportion of the birds which survived the inoculation resisted fowl-plague.

Two birds were immunized with a bromoform virus, but this could not be repeated in spite of frequent attempts.

All the immune fowls used in these experiments were first tested by inoculation with the strain with which they had been vaccinated, before being tested with a different strain of virus.

Test Dose of Virus.

In all these experiments the test dose of virus was severe. It consisted of 0·25 c.c. of an emulsion made in 5·0 c.c. of saline with a fragment of liver, the size of a pea, taken from a bird dead of fowl-plague. This was inoculated intramuscularly. A healthy bird was in each case used as a control.

Results.

"Rice"	Immune	bird	689	resisted	Palestine	virus.
"	"	"	811	"	Egyptian	"
"	"	"	477	"	"Ex."	"
Palestine	"	"	1128	"	"Bax."	"
"	"	"	955	"	Egyptian	"
"	"	"	1020	"	"Rice"	"
"Bax."	"	"	1178	"	Egyptian	"
"	"	"	1949	"	French	"
"	"	"	1964	"	Dutch	"
"	"	"	1277	"	"Rice"	"
"	"	"	1154	"	Palestine	"
Egyptian	"	"	224	"	"Rice"	"
"	"	"	225	"	Palestine	"
"Ex."	"	"	1089	"	"	"
"	"	"	1091	"	"Bax."	"

In one case, however, Bird 650, immunized with the Palestine strain, succumbed to inoculation with the "Ex." virus. This case, and three others in which the degree of immunity appeared to wane after a long lapse of time, are discussed in the next part of this paper.

DURATION OF FOWL-PLAGUE IMMUNITY IN FOWLS.

The following observations on four fowls are of interest, as they indicate that the immunity to fowl-plague is of short duration. Each of these fowls at one time resisted inoculation with fowl-plague virus, but died on subsequent testing, having been allowed a long interval of "rest."

Fowl 1062.

This fowl was inoculated with an emulsion of known pH for testing the survival of the virus. It did not die, and on testing a week later with the same virus as had been used in the emulsion, it resisted infection. Thirty-two days afterwards it was again inoculated with this virus, and died in 48 hours.

Fowl 1128.

This fowl was a Palestine immune bird which also resisted inoculation with the "Bax." strain of virus. Fifty-two days after the last inoculation it was successfully infected with the Palestine strain, and died in two days.

Fowl 650.

Fowl 650 was immunized by Todd's method, using the Palestine virus. Sixty-two days later it was inoculated with the "Ex." virus, and died of fowl-plague in two days.

Fowl 1178.

This was an "Ex." immune fowl which a week after the immunization resisted inoculation with the Egyptian virus. After the lapse of 154 days it succumbed to infection with the Rice virus.

CONCLUSIONS.

(1) Using seven strains of fowl-plague virus, no evidence of the occurrence of immunologically different types was found.

(2) It was found that fowls known to be able to resist inoculation with fowl-plague virus may, after a lapse of 32 days or more, succumb to fowl-plague if then inoculated intramuscularly with the virus.

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THE FILTERABILITY OF VACCINIA VIRUS.

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Received for publication May 19th, 1931.

OWING to the curiously discrepant character of the evidence relating to the filterability of the virus of vaccinia, it has not been possible until quite recently to give a categorical answer to the question whether this virus is filterable at all through filters that retain bacteria and, if filterable, to what extent its potency is depreciated in the process. A very fair critique of the position as it stood in 1929 is given by Blaxall (1930), and to this, as also to Gildmeister's review (1927) in the handbook of Lentz and Gins, the reader must be referred for historical details. Blaxall states that he, like one of us (Green, 1914), had in the past failed to secure potent filtrates of vaccinia, while taking advantage of every manœuvre believed to be conducive to success. He pointed out that " though success of a kind had been obtained from time to time by different observers, there was a freakishness about the results that rendered them far from convincing." Before his death he had tested the method advocated by Ward (1929), to whose work we refer below, but again with a negative result. In an earlier critique of this question by Ledingham (1926), whose own experiments with British Berkefeld and Seitz filters had also yielded negative results, the conclusion was reached that the question of the filterability of vaccinia was still an open one. In many of the experiments in which successful results with filtrates were claimed, the tests for potency of filtrates were not calculated to supply convincing evidence. At the most it could be said that where a filtrate gave a positive result, its titration revealed a great loss of virus in the process of filtration.

The experiments recorded here were commenced by one of us (Green) over twelve months ago in the endeavour to follow the method prescribed by Yaoi and Kasai (1929). His results with freshly harvested sheep-lymph were so completely confirmatory of those of the Japanese workers that it was decided to extend the inquiry by employing other races of vaccine virus, and by testing out also the method of Ward (1929). Control tests were also carried out in which the various prescriptions recommended were not adhered to.

Yaoi and Kasai (1929) started from the assumption that difficulties in securing potent filtrates of vaccinia were due to adsorption of the virus by the filter, and might be surmounted by altering the pH of the fluid passed through. Freshly scraped calf lymph was diluted 1 in 100 with distilled water and passed through filter-paper. When the resulting suspensions were adjusted to pH 8 before filtration through Berkefeld V filters the filtrates were active, whereas filtrates from suspensions adjusted to pH 6–7 contained little or no virus.

They infer from this successful experiment that negative results with kept lymph are due to the acid reaction which develops in such lymph. Later the Japanese workers found that if the adsorptive powers of the filter were satisfied by preliminary passage through it of an acid solution of egg-white—a procedure employed by Holderer (1912) to facilitate filtration of enzymes—it was possible for the virus suspension to pass through V and N filters with its potency in great part conserved. The filtrates were tested on the scarified skin of calves.

Ward and Tang (1929), in their first paper, state that they applied to vaccinia the filtration procedure which they had found successful with the virus of herpes. Fresh scrapings from the vaccinated calf were carefully ground and added to hormone broth. After centrifugalization, the virus suspended in hormone broth was passed through V filters and, although there was considerable loss by adsorption, tests on the scarified skin of the rabbit revealed the presence of potent virus. Ward's second paper (1929) contains protocols and titrations of filtrates obtained by his method. The filtrates were tested by intradermal inoculation of rabbits. Importance is attached to the following points: The scrapings used are fresh; the pulp is ground in a glass mortar with fragments of pyrex glass; hormone broth is added to make a 5 per cent. emulsion of the pulp; the suspension is centrifuged for 30 minutes at 2500 revolutions per minute; the supernatant fluid is then passed through a V filter at a negative pressure of 30 cm. of mercury. With regard to filters, the Berkefeld V was the best, the Berkefeld N allowed only a little virus through, while the Chamberland L 1 (*bis*) failed. Titration showed that filtrates were 16 times less active than the unfiltered controls, and it would appear from examination of the protocols that the intensity of the reactions with filtrates fell off rather rapidly as dilution increased.

In further experiments on similar lines by Tang (1930) it was found that N filters did not allow the virus through. Both he and Ward also demonstrated that when potent filtrates were secured, centrifugalization of the filtrates revealed a concentration of virus in the lower layers of the suspensions, and the conclusion was reached that the virus particles must be of considerable size.

TESTS OF THE JAPANESE METHOD BY A. B. GREEN AT THE INSTITUTE LYMPH STATION.

Method.

Freshly scraped vaccine pulp was ground very finely and a 1 per cent. suspension prepared with distilled water. This was filtered through paper. The pH was adjusted in six experiments of the series to 6·4, 6·6, 6·8, 7·0, 8·2 and 8·4.

White of egg was added to 6 times its volume of water. The emulsion after adjustment to pH 6·4 was filtered through paper.

Water was drawn through Berkefeld V and N filters and followed by 15 c.c. of the egg-white solution, and then by 15 c.c. of the virus suspension.

B. prodigiosus was added to each suspension and tested for in the filtrate. The filtrates and the unfiltered controls were inoculated into lightly made cutaneous incisions on the shaved skins of rabbits and sheep and the results were read on the 3rd or 4th day.

Results.

In all six experiments of this kind the undiluted filtrates (which alone were tested) gave very definite but varying degrees of vesiculation of characteristic type. Filtrates from the N filters gave much weaker reactions. So far as these tests went, the variation in the initial pH of the suspensions did not appear to influence the result.

Titration of filtrates by intradermal test on rabbits.—From six filtrates prepared by the method described above, successive dilutions down to 1 in 10^{-6} were made and inoculated in 0·2 c.c. quantities intradermally. Caliper measurements of the reactions were not taken, consequently the actual protocols are not detailed, but definite reactions were noted in all cases down to the highest dilution of one in a million.

EXPERIMENTS CARRIED OUT AT THE LISTER INSTITUTE, LONDON.

In the following experiments, carried out by one of us (G. H. E.), two sources of virus material were investigated—sheep lymph and neuro-testicular virus. Distilled water and hormone broth were used to dilute the virus, and their values compared from the standpoint of assisting filterability.

English Berkefeld, Berkefeld "V" and Berkefeld "N" filters were used. These were tested from time to time by using a young broth culture of *B. prodigiosus*. Filtration was carried out under low pressure. All filtrates tested in these experiments were bacteriologically sterile.

Unfiltered and filtered material in 0·2 c.c. amounts were tested intradermally on the same rabbit, and measurements by calipers taken daily from 3 to 6 days. The reaction was found to be at its height on the 5th and 6th days.

Sheep Lymph.

Fresh lymph was ground as finely as possible without sand in a mortar and diluted 1 in 50, either with distilled water or hormone broth. It was then passed through coarse filter-paper and the pH adjusted to the required point. Both lined and unlined filters were used. Where unlined filters were used the virus was passed through directly, but in lined filters 20 c.c. of acidified egg-white (pH 6·4), filtered through paper, were passed through the filter immediately before the virus.

Table I shows the effect on virus activity of filtering through an unlined Berkefeld "V" filter when the pH of the virus had been adjusted from 6·4 to 8·4. Distilled water was used to dilute the virus.

TABLE I.—*Unlined V Filter.*

Dilution of virus.	Filtered virus.								Control.	
	pH 6·4.		pH 7·0.		pH 7·4.		pH 8·4.		Unfiltered virus.	
	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.
1 in 50	.20 mm.	.25 mm.	.20 mm.	.25 mm.	.18 mm.	.18 mm.	.20 mm.	.20 mm.	.25 mm.	.30 mm.
10^{-2}	.20 ,,	.25 ,,	.20 ,,	.25 ,,	.16 ,,	.16 ,,	.15 ,,	.15 ,,	.20 ,,	.25 ,,
10^{-3}	.15 ,,	.20 ,,	.15 ,,	.20 ,,	.15 ,,	.15 ,,	.15 ,,	.15 ,,	.18 ,,	.20 ,,
10^{-4}	.10 ,,	.10 ,,	.10 ,,	.10 ,,	.10 ,,	.10 ,,	.8 ,,	.8 ,,	.10 ,,	.15 ,,
10^{-5}	.5 ,,	.5 ,,	.5 ,,	.5 ,,	.5 ,,	.5 ,,	n.	n.	.5 ,,	.5 ,,
10^{-6}	n.	n.	n.	n.	n.	n.	n.	n.	n.	n.

Caliper measurements in millimetres have been averaged for each reaction whenever irregularity occurred, and indicate the height of reaction on the 5th and 6th days. This method of measurement was used throughout.

Table II shows the effect of filtering the same virus material as in Table I through a lined Berkefeld V filter.

TABLE II.—*Lined V Filter.*

Dilution of virus.	Filtered virus.								Control.	
	pH 6·4.		pH 7·0.		pH 7·4.		pH 8·4.		Unfiltered virus.	
	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.
1 in 50	.25 mm.	.25 mm.	.23 mm.	.25 mm.	.20 mm.	.20 mm.	.20 mm.	.20 mm.	.25 mm.	.25 mm.
10^{-2}	.20 ,,	.20 ,,	.20 ,,	.20 ,,	.20 ,,	.20 ,,	.20 ,,	.20 ,,	.20 ,,	.20 ,,
10^{-3}	.18 ,,	.18 ,,	.15 ,,	.15 ,,	.15 ,,	.15 ,,	.12 ,,	.12 ,,	.18 ,,	.18 ,,
10^{-4}	.10 ,,	.10 ,,	.10 ,,	.10 ,,	. 8 ,,	. 8 ,,	.10 ,,	.10 ,,	.10 ,,	.10 ,,
10^{-5}	. 5 ,,	. 5 ,,	. 5 ,,	. 5 ,,	n.	n.	. 5 ,,	. 5 ,,	. 5 ,,	. 5 ,,
10^{-6}	n.	n.	n.	n.	n.	n.	n.	n.	n.	n.

From Tables I and II it was apparent that very little, if any, virus was lost when passed through a Berkefeld V filter, and that adjustment of pH within the range investigated was not of importance. Lining the filter did not increase filterability of virus.

In order to test further the filterability of the virus a quantity of the same virus was passed through an English Berkefeld and a Berkefeld N filter. Table III shows that the greater part of the virus was retained in both types of filter. No adjustment in pH was made, and the filters were unlined.

TABLE III.

Dilution of virus.	English Berkefeld.		Berkefeld N.		Unfiltered control.	
	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.
1 in 50	5 mm.	5 mm.	15 mm.	15 mm.	25 mm.	25 mm.
10^{-2}	5 ,,	5 ,,	10 ,,	10 ,,	20 ,,	20 ,,
10^{-3}	n.	n.	5 ,,	5 ,,	15 ,,	15 ,,
10^{-4}	n.	n.	n.	n.	10 ,,	10 ,,
10^{-5}	n.	n.	n.	n.	5 ,,	5 ,,
10^{-6}	n.	n.	n.	n.	n.	n.

It was thought possible that lining the English Berkefeld and N filters might increase their permeability. The experiment was repeated, therefore, using lined filters. The results showed that the English Berkefeld filter, whether lined or unlined, permits only a small quantity of virus to pass through, and that the Berkefeld N allows only slightly more virus to pass than the English Berkefeld.

Hormone Broth.

Fresh sheep lymph and a fresh passage strain of neuro-testicular virus were used. Sheep lymph was prepared as in the previous experiments, except that hormone broth was used as diluent. Neuro-testicular virus was prepared by grinding a fresh 4-day infected testicle and diluting 1 in 50 with hormone broth. Both strains of virus were passed through filter-paper. No adjustment in pH was made and lined and unlined filters were tested. English Berkefeld, Berkefeld V and N filters were used.

Table IV shows the filterability of sheep lymph, and Table V that of neuro-testicular virus.

TABLE IV.—*Filterability of Sheep Lymph in Hormone Broth.*

Dilution of virus.	English Berkefeld.		Berkefeld V.		Berkefeld N.		Unfiltered control.	
	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.
1 in 50	20 mm.	20 mm.	35 mm.	35 mm.	15 mm.	15 mm.	35 mm.	35 mm.
10^{-2}	13 ,,	13 ,,	30 ,,	30 ,,	10 ,,	10 ,,	30 ,,	30 ,,
10^{-3}	n.	n.	18 ,,	18 ,,	n.	n.	18 ,,	18 ,,
10^{-4}	n.	n.	10 ,,	10 ,,	..	..	9 ,,	9 ,,
10^{-5}	n.	n.	n.	n.	..	..	5 ,,	5 ,,
10^{-6}	n.	n.	n.	n.	..	..	5 ,,	5 ,,

TABLE V.—*Filterability of Neuro-Testicular Virus in Hormone Broth.*

Dilution of virus.	English Berkefeld.		Berkefeld V.		Berkefeld N.		Unfiltered control.	
	5th day.	6th day.	5th day.	6th day.	5th day.	6th day.	5th day.	6th day
1 in 50	25 mm.	25 mm.	30 mm.	30 mm.	22 mm.	22 mm.	35 mm.	35 mm.
10^{-2}	10 ,,	10 ,,	25 ,,	25 ,,	20 ,,	20 ,,	25 ,,	25 ,,
10^{-3}	n.	n.	15 ,,	15 ,,	15 ,,	15 ,,	22 ,,	22 ,,
10^{-4}	n.	n.	12 ,,	12 ,,	5 ,,	5 ,,	20 ,,	20 ,,
10^{-5}	n.	n.	10 ,,	10 ,,	n.	n.	15 ,,	15 ,,
10^{-6}	n.	n.	8 ,,	8 ,,	n.	n.	10 ,,	10 ,,
10^{-7}	n.	n.	n.	n.	n.	n.	5 ,,	5 ,,

The effect of passing neuro-testicular virus in hormone broth through lined filters was investigated. The results showed that there was no increase in filterability by using lined filters instead of unlined filters.

The effect of freezing sheep lymph and neuro-testicular virus over a considerable period before filtering was investigated. It was found that this did not alter filterability to any appreciable extent, either when diluted with distilled water or hormone broth.

STORAGE OF FILTRATES.

The effect of storing filtrates for a period of 1 to 3 weeks in the dark at 35° F. was investigated. At the end of this period they were tested intradermally on rabbits. The filtrates remained active, and showed a definite but only slight deterioration when compared with their original titre.

A hormone broth filtrate of neuro-testicular virus was kept for one week at cold room temperature and tested intradermally on rabbits. Table VI shows that this period of storage had no appreciable deleterious effect.

TABLE VI.—*Hormone Broth Filtrate kept in Cold Room.*

Dilution of virus.	Berkefeld V filtrate. Original titre.		After storing 1 week in cold room.	
	5th day.	6th day.	5th day.	6th day.
1 in 50	25 mm.	30 mm.	25 mm.	28 mm.
10^{-2}	25 ,,	30 ,,	25 ,,	25 ,,
10^{-3}	20 ,,	20 ,,	20 ,,	20 ,,
10^{-4}	15 ,,	18 ,,	15 ,,	15 ,,
10^{-5}	12 ,,	12 ,,	10 ,,	10 ,,
10^{-6}	8 ,,	8 ,,	5 ,,	5 ,,
10^{-7}	n.	n.	n.	n.

Experiments were made to determine whether heavier suspensions were practicable for filtration. A 1 in 10 finely-ground suspension in distilled water was used for this purpose. For comparison a concentration of 1 in 1000 was made at the same time. After filtering through paper the heavier suspension passed readily through a Berkefeld V filter under low pressure. The filtrates were tested without dilution by scarification on rabbits. The results of these experiments revealed, on the whole, surprisingly little difference in the filterability of the lighter and heavier suspensions.

DISCUSSION.

In view of the many attempts made in the past to secure potent filtrates of vaccinia virus and the variable results obtained, it is difficult to explain, in the light of the results recorded in this communication, the discrepancy in the earlier work. Generally, filtrates were weak, and gave unsatisfactory reactions. In some cases concentration of the filtrate was necessary in order to demonstrate the presence of virus in it. From the present work it is clear that the lining of filters with acidified egg-white as employed by Yaoi and Kasai is unnecessary, and that adjustment of pH of the virus need not be employed. The virus passes through readily when either acid or alkaline. The Berkefeld V filter is the most satisfactory of those employed in this inquiry for filtering vaccinia virus. The virus passes readily through this filter without great loss in potency. While different filters of the same type vary considerably, it can be stated as a general rule that 90 per cent. of the virus passes through the V filter when determined by caliper measurements of intradermal rabbit reactions. Such measurements are considered necessary, since the amount of virus passing through a filter can be assessed, by comparison with the ulfiltered control, only in terms of relative intensity of reaction to the same dilution.

A large proportion of the virus is adsorbed by Berkefeld N filters, and an even greater proportion by the English Berkefeld filter. This has been assessed by passing the same amount of virus through each type of filter employed. Seitz filters, in so far as they were investigated, proved unsatisfactory in that high pressure was necessary to secure a filtrate, and it was not possible to secure a filtrate free from bacteria.

The discrepant results displayed in earlier work may be, at least in part, accounted for by the use of scarification as a means of testing the activity of filtrates. It is now generally accepted that the intradermal test possesses considerable advantage over the scarification method, in that it is possible to inject a measured amount which is practically entirely retained in the dermis. Where water-clear filtrates are concerned, which tend to run off scarified areas, loss of virus is entirely avoided by the intradermal method. Accurate measurements of resulting reactions can be made. A higher titre of the virus is invariably obtained by the intradermal method of inoculation.

When hormone broth is used to dilute the virus material the virus is readily filterable; as shown by comparison of Tables I and IV, provided that quantities as large as 20 c.c. of virus material pass through the filter, there does not appear to be any advantage in using hormone broth in place of distilled water. When small amounts of virus are filtered it seems probable that hormone broth or lining the filter may be advantageous.

It is possible that harvesting the virus material early may have some influence on its filterability. Elementary bodies are present in larger numbers earlier in the reaction than at later periods. It would be of interest to investigate to what extent the disposition of these bodies in the lesion may influence the filterability of vaccinia virus.

The fact that lymph may be obtained bacteria-free and of high potency by ordinary filtration methods is important both from the standpoint of virus study and of improved vaccination practice.

CONCLUSIONS.

Vaccinia virus passes readily through Berkefeld V filters, and to some, though much less extent, through Berkefeld N and English Berkefeld filters.

The pH of the virus material does not seem to be important within the range of 6·4 to 8·4.

Lining the filters with acidified egg-white does not apparently enhance the filterability of large quantities of virus. It has not, however, any deleterious effect.

Individual filters of the same type vary to a considerable degree in the amount of virus which passes through.

Hormone broth does not appear to be superior to distilled water as a diluent of virus material before filtering, when large quantities of virus are filtered.

Filtrates may be kept for some time in the cold without marked deterioration in activity.

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